Antifungal activity of endophytes from *Arctotis arctotoides* (L.F.) O. Hoffm against *Pythium* and *Rhizoctonia* root-rot diseases of maize (*Zea mays* L.).

By

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DISSERTATION SUMMARY

Maize (*Zea mays* L.) is one of the predominant crops worldwide, together with wheat (*Triticum aestivum* L.) and rice (*Oryza sativa* L.). Approximately 60% of maize produced in South Africa is white maize and is a staple food for many South Africans. About 40% of maize grown is yellow maize and is used for animal feed. About 73% of maize produced in South Africa is produced in the Free State, Mpumalanga, and North West provinces. Maize is grown under various climatic conditions, which sometimes become favourable for disease development. Various plant pathogens such as-, *Fusarium* spp., *Rhizoctonia solani*, and *Pythium* spp. cause diseases in maize. These diseases are usually controlled by cultural practices and fungicides-. However, these control strategies are not always effective, especially against root rot pathogens. Infection of maize plants by *Pythium* spp. causes brown root lesions, whereas *R. solani* causes dark-brown root lesions.

As an alternative to cultural and chemical control, biological control can be used to mitigate plant diseases. Biological control is based on the premise that the biocontrol agents (BCAs) produce antimicrobial compounds that inhibit pathogens' growth. BCAs also compete with pathogens for resources in the host plant and rhizosphere. Some BCAs induce systemic acquired resistance in host plants. Endophytes are microorganisms that dwell within tissues in their host plants without any visible symptoms, and can be used as BCAs against plant diseases.

Medicinal plants are host to a distinctive microbiome and are an excellent source of bioactive compounds which can be applied in agriculture, medical and pharmaceutical fields. Previous studies have shown that endophytes from medicinal plants are involved in producing secondary metabolites in their host plants. These endophytes impact the functioning of antioxidant enzymes, resulting in activated defence mechanisms against pathogens. *Arctotis arctotoides* (L.f) O. Hoffm is a medicinal plant used as pastes or decoctions against wounds, epilepsy, ringworms and other ailments. There are no reports where this medicinal plant has been tested against plant pathogens hence this study is necessary. In this study, endophytes isolated from *A. arctotoides* were tested against *R. solani* and *Pythium* spp. root rot pathogens of maize. This is based on the premise that endophytes isolated from this plant will inhibit the growth of plant pathogens.

Disease-free *A. arctotoides* plants were collected from various locations in the Eastern Cape Province, Republic of South Africa, and transported to the laboratory to isolate bacterial endophytes. Twenty-six (26) bacterial endophytes were isolated from the roots, stem, and leaves within 24 hours of sampling. These endophytes were screened *in vitro* for their antifungal activity against *R. solani* and *Pythium* spp. root pathogens of maize. The endophytes were identified using Internal Transcribed Spacers (ITS) sequencing. Results of the *in vitro* screening showed that ten bacterial endophytes were antagonistic to *R. solani*, whereas-, 11 were antagonistic to *Pythium* spp. The percentage inhibition ranged from 17-50% and 8-64% for *R. solani* and *Pythium* spp. respectively. Only three bacterial endophytes (*Bacillus cereus* NYR11, *Morganella morganii* L143 NYR3, and *M. morganii* KC-Tt-01 NYL20) inhibited the growth of both pathogens significantly.

The antagonistic effect of the best ten bacterial endophytes against each root rot pathogen was further evaluated under greenhouse conditions. The bacterial endophytes were applied as seed treatments and pathogens inoculated in the rhizosphere except the control treatments. The parameters measured were: -plant height once a week for six weeks, root length, number of root lesions, root and shoot weight at harvesting. Maize plants treated with the endophytes *Bacillus cereus* NYR11, *Proteus mirabilis* NYR9, and *Morganella morganii* strain DG56-16 NYS3 against *R. solani* and *Myroides odoratus* strain 6G NYL18, *Alcaligenes faecalis* NYS7, and *Ralstonia* spp. NYR8 against *Pythium* spp. showed low numbers of root lesions, increased root length, root and shoot weights. These bacterial endophytes showed potential to be used as BCAs against *R. solani* and *Pythium* spp.

The antagonistic effect of the best three bacterial endophytes against each pathogen was further evaluated as mixtures in the greenhouse. These were *B. cereus* NYR11, *P. mirabilis* NYR9, and *M. morganii* DG56-16 NYS3 against *R. solani* and *M. odoratus* strain 6G NYL18, *A. faecalis* NYS7, and *Ralstonia* spp. NYR8 against *Pythium* spp. The mixtures were applied as seed treatments and pathogens inoculated in the rhizosphere except the control treatments. The parameters measured were-, plant height once a week for six weeks, root length, number of root lesions, root and shoot weight at harvesting. *B. cereus* NYR11 + *M. morganii* DG56-16 NYS3, and *P. mirabilis* NYR9 + *M. morganii* DG56-16 NYS3, significantly reduced the number of root lesions, increased root length and root weight in the presence of *R. solani.* In maize plants

inoculated with *Pythium* spp. the single applications of *Ralstonia* spp. NYR8 and *M. odoratus* 6G NYL18 were better treatments than mixtures. These endophytes, especially *M. odoratus* 6G NYL18 increased root length, root and shoot weight, reduced the number of root lesions when applied individually. The *M. odoratus* 6G NYL18 + *A. faecalis* NYS7 + *Ralstonia* spp. NYR8 mixture was a better treatment than other mixtures, even though it was not better than the single application *M. odoratus* NYL18.

The potential mode of action of the best three endophytes against each pathogen were evaluated. Modes of action assessed in this study were siderophore production, protein, chitin, and cellulose degradation. Out of six bacterial endophytes evaluated, only *Ralstonia* spp. NYR8 did not produce cellulase and siderophores. *P. mirabilis* NYR9 and *M. odoratus* 6G NYL18 did not produce protease. All the bacterial endophytes were unable to degrade chitin. Other modes of action used by the bacterial endophytes against the pathogens can be further evaluated.

DECLARATION

I, Ncumisa Yekelo, declare that:

i. The research reported in this thesis except otherwise indicated is my original work;

ii. This dissertation has not been submitted for any degree or examination at any other university

iii. This dissertation does not contain other person's data, pictures or graphs or other information, unless specifically acknowledged as being sourced from other persons

iv. This dissertation does not contain other person's writing unless specifically acknowledged as being sourced from other researchers. Where other written sources have been quoted, their words have been re-written, but the general information attributed to them has been referenced.

v. This dissertation does not contain text, graphics or tables copied and pasted from the Internet unless specifically acknowledged and the source being detailed in the thesis and in the References section.

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Finally, I would like to thank God for His love and grace in my life.

DEDICATION

This study is dedicated to my aunt, Mvulakazi Yekelo, thank you for your undying love, support and prayers. I am because you are, literally.

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DISSERTATION INTRODUCTION

Maize (*Zea mays* L.) is one of the most important cereals as it is a staple food for over one billion people (Suleiman *et al*., 2017). Maize is normally produced during the summer months when the average temperature is above 23°C and cannot be produced in areas where the average daily temperature is below 19°C (Du Plessis *et al*., 2003). Developing countries depend on maize as a staple food and it is consumed in its different forms such as breakfast cereals, processed starch and directly as grains. In developed countries maize can also be used in its processed form as fuel (Du Plessis *et al*., 2003). At least 60% of maize produced in South Africa is white maize used for human consumption (DAFF, 2017).

As maize is produced in various climatic conditions, biotic and abiotic stresses may reduce yield quality and quantity (Meissle *et al*. 2010). Different microorganisms such as bacteria, fungi, viruses and nematodes cause plant diseases (Agrios, 2005). The most common diseases of maize are caused by *Fusarium*, *Pythium*, and *Rhizoctonia* species (Ncube *et al*., 2011). In maize, *Pythium* and *R. solani* diseases are usually associated with seedlings but adult plants may also be infected. Adult plants may be stunted and die as a result of root lesions that are caused by the pathogen on the roots and stems of adult plants (Agrios, 2005).

Usually, cultural practices and fungicide seed treatments may be used to reduce losses as a result of root rot pathogens. However, these control strategies are not always effective and consistant use of fungicides may lead to fungicide resistance (Jose and Christy, 2013). Fungicides are not regarded as eco-friendly and may be hazardous to human beings. Therefore, alternative control strategies such as biological control are being considered. Endophytes can be defined as microorganisms that are able to live asymptomatically within their host tissues and each plant species is regarded as a host to at least one endophyte (Jose and Christy, 2013).

Since endophytes inhabit the host plant with no visible symptoms, they have the ability to inhibit pathogen growth through competition (Hassan, 2017) and by releasing metabolites that induce host plant defence mechanisms (Martinez-klimova *et al*., 2017). For years medicinal plants have been used for the well-being of humans and

as an important reserve for pharmaceuticals (Palanichamy *et al*., 2018). Therefore, it has been suggested that endophytes found in medicinal plants may produce natural bioactive products (Liu *et al*., 2016). Since they are regarded as a repository of endophytes (Huang *et al.*, 2007), medicinal plants may be used as novel antimicrobial substances (Khan *et al*., 2018).

Arctotis arctotoides (L.f) O. Hoffm, which belongs to the Asteraceae family is a common herbaceous decumbent medicinal plant in the Eastern Cape, Republic of South Africa. The plant grows as a roadside weed in coastal and summer rainfall areas of South Africa. According to the indigenous people of the Eastern Cape, extracts from *A. arctotoides* are used for the treatment of diseases such as epilepsy, indigestion, catarrh of the stomach, ringworm, wounds, pimples and insect bites (Afolayan, 2003; Afolayan *et al*., 2007; Badmus and Afolayan, 2012; Dlova and Ollengo, 2018). It has been shown that root and shoot extracts are able to inhibit the growth of some bacterial and fungal human pathogens e.g. *Cladosporium herbarum* which causes asthma attack (Afolayan, 2003). Research shows that extracts from *A. arctotoides* have antifungal activity against the growth of *Alternaria alternata*, *Aspergillus niger* (Afolayan *et al*., 2002), *A. flavus* and *Penicillium digitatum* (Afolayan, 2003).

The aims of this study were to isolate endophytes from the leaves, stem, and roots of *A. arctotoides* and screen their antifungal activity against *Pythium* spp. and *R. solani* root rot pathogens of maize.

Research objectives:

The specific research objectives of the study include:

1. To collect healthy *A. arctotoides* plants from various regions in the Eastern Cape, Republic of South Africa, isolate endophytes and screen them against *Pythium* spp. and *R. solani* root rot pathogens of maize *in vitro*;
2. To evaluate selected bacterial endophytes against *Pythium* spp. and *R. solani* root rot pathogens under greenhouse conditions;
3. To evaluate the best three bacterial endophytes and their combination against each pathogen under greenhouse conditions and screen for their potential modes of actions.

This dissertation has been written in the form of four chapters. Each chapter is focused on a specific objective of the research that was conducted. With an exception of Chapter one, “literature review”, the other three chapters were independent studies and were written in the form of research chapters. Each chapter is following the format of a stand-alone research paper. This format is the standard dissertation model that has been adopted by the University of KwaZulu-Natal because it facilitates the publishing of research out of the dissertation far more readily than the older monograph form of dissertation. As such, there is some unavoidable repetition of references, methods and some introductory information between chapters.

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Chapter 1: Literature Review

1.1 Introduction

Since its first discovery in Mexico, maize (*Zea mays* L.) has been produced throughout the cropping world and is one of the most highly cultivated cereal crops (Edmeades *et al*., 2017). It is believed that maize was derived from wild grass about 7000 years ago (Ranum *et al*., 2014). Mexico has different climatic conditions making it easier for maize to be genetically modified, leading to new landraces with better adaptability under different environmental conditions across the world (Edmeades *et al*., 2017). Maize is the most crucial crop used as a cereal globally and is the most important staple food for over one billion people (Suleiman *et al*., 2017). Maize, wheat (*Triticum aestivum* L.) and rice (*Oryza sativa* L.) are the most important crops globally and serve as staple food in various countries, especially developing countries (Shiferaw *et al*., 2011).

1.1.1 Maize production

The maize plant requires specific environmental conditions and nutrient uptake for optimum yields. About 450 to 600mm of water is needed in a season to obtain reasonable yields, and this water is extracted chiefly from the soil moisture reserves through the crop's roots. The uptake of the most important nutrients for a maize plant is maximised at the flowering stage. At maturity, the uptake of a single maize plant is 8.7g of Nitrogen, 5.1g of Phosphorous, and 4.0g of Potassium. About 15.0-18.0kg of Nitrogen, 2.5-3.0kg of Phosphorous, and 3.0-4.0kg of Potassium are removed from the soil per ton of grain produced (Du Plessis, 2003).

Maize is usually produced during the summer months when the average temperature is above 23°C and cannot be produced in areas where the average daily temperature is less than 19°C (Du Plessis, 2003). For optimum germination, the crop requires soil temperatures of 18-19°C even though germination can still occur at lower temperatures of 10°C (Mbotho, 2018). Even though maize can tolerate warm temperatures, above 32°C, the total yield obtained will be reduced. The maize plant is vulnerable to frost damage across all growth stages, therefore, a frost-free period of 120 to 140 days is required to prevent damage (Du Plessis, 2003).

1.1.2 Economic importance of maize

Approximately 875 226 630 tons of maize were produced worldwide in 2012 and the leading producers included the United States, China and Brazil with a total yield of 31%, 24%, and 8% respectively. In Africa, maize is produced on a larger scale than other crops as illustrated in Table 1.1 (Macauley, 2015). In Sub-Saharan Africa, it is a staple diet where 95% of the production is used for human consumption and as a source of income in poorly developed areas (Shiferaw *et al*., 2011).

Table 1.1: Major staple foods in Africa (Macauley, 2015).

Crop	**Area (ha)**	**Production (ton)**
Maize	34,075,972	70,076,591
Wheat	10,224,952	24,704,201
Sorghum	23,142,595	23,350,064
Rice	11,206,813	28,798,202
Millet	19,998,008	16,008,838

Compared to other African countries, South African maize production is the largest (Mulungu and Ng'ombe, 2020). Maize is the most dominant grain crop produced in South Africa and is grown in different environmental conditions in the country. About eight million tons of maize are produced in close to 3.1 million ha of land yearly in South Africa (Du Plessis, 2003). At least 60% of maize produced in South Africa is white maize used for human consumption (DAFF, 2017). The remaining 40% is yellow maize used mainly as livestock feed (DAFF, 2017). Developing countries depend on maize as a staple food, and it is consumed in its different forms such as breakfast cereals, processed starch, and directly as grains. In developed countries maize can also be used in its processed form as fuel (Du Plessis, 2003).

Approximately 600 000 households rely on subsistence farming in South Africa and with maize being a staple food, the amount of maize consumed by one person may be 300g a day (Ncube *et al.*, 2011). According to the World Health Organisation (WHO), maize consumption (g/ person/ day) in South Africa is the fifth in Africa following Lesotho, Malawi, Zambia, and Zimbabwe (Ranum *et al*., 2014).

1.1.3 Production constraints

As maize is produced under various environmental conditions, production is limited by different factors depending on the environment in which the crop is grown. Insect pests, weeds, and pathogens greatly reduce yield and quality of maize even though pesticides and fungicides are used to lower the amount of yield losses (Meissle *et al*. 2010). Factors such as low soil fertility, irregular rainfall, pest infections, poor infrastructure, marketing, and policy restrictions primarily reduce the production of maize (Odendo, De Groote & Odongo, 2001). Climate change has primarily influenced the agriculture system in the world; as a result, increasing daily temperatures and changing rainfall patterns are experienced more frequently than before. Lack of rainfall leads to difficulty in crop production as freshwater is required for crops to produce optimum yields (Elliott *et al*., 2014). Reduced soil fertility is a vital constraint in maize production as yield losses from 10-50% have been estimated due to low nitrogen levels in the soil (Ribeiro *et al*., 2017).

1.1.3.1 Maize diseases

Different microorganisms such as fungi, bacteria, viruses, and fungal-like organisms (oomycetes), cause plant diseases (Agrios, 2005). Due to financial constraints, subsistence farmers' use retained seeds to plant in the new season which may encourage plant pathogenic infections. Other cultural practices such as maize monoculture, may lead to increased fungal inoculum and pest damage, causing severe fungal infections such as stem, ear, and root rot in crops (Ncube *et al.,* 2011). The most common maize diseases are caused by plant pathogens including *Rhizoctonia, Pythium*, *and Fusarium* species.

Plant diseases are of importance to maize producers as they may lead to yield losses of up to 30% in some countries or in severe epidemics, wipe out maize fields. Maize diseases such as ear and kernel infections caused by *Fusarium*, *Aspergillus* and *Stenocarpella* species lead to substantial yield losses in maize fields since they reduce grain and yield quality and increase mycotoxin contaminations (Mukanga *et al*., 2011).

With maize quality and yield being the most important traits for commercial farmers, these farmers apply management strategies that decrease losses. On the contrary, subsistence farmers are unable to implement these strategies due to shortfall on the required resources to maintain the quality of grain produced. Because of their

undersupply in hybrid seed, fertilisers and pesticides, subsistence farmers have poor yield and quality which is a result of poor soil fertility, pathogenic infections and pest damages (Ncube *et al.*, 2011).

1.2 Pythium and *Rhizoctonia* plant disease

1.2.1 Disease cycle and epidemiology

1.2.1.1 *Pythium*

Pythium is an oomycete pathogen that causes damping-off, root and seed rots. *Pythium* is most common on young plants, but when adult plants are infected, they may be stunted and die, leading to huge yield losses. This is because of the lesions induced by the pathogen on the roots and stems of infected adult plants. Infected seeds fail to germinate because once in contact with *Pythium* they become mushy, turn brown, shrivel, and finally rupture. This pathogen can also cause pre-emergence damping-off in which the seeds are colonized before emergence leading to invaded cells of the seeds collapsing. Emerged seedlings are infected at the roots and stems, and when infestation is high, they become water-soaked, discoloured and die. The invaded basal part of the plant becomes thinner, and so the whole plant collapses. In most cereals and turf grasses, infection by *Pythium* may cause *Pythium* blight (Agrios, 2005).

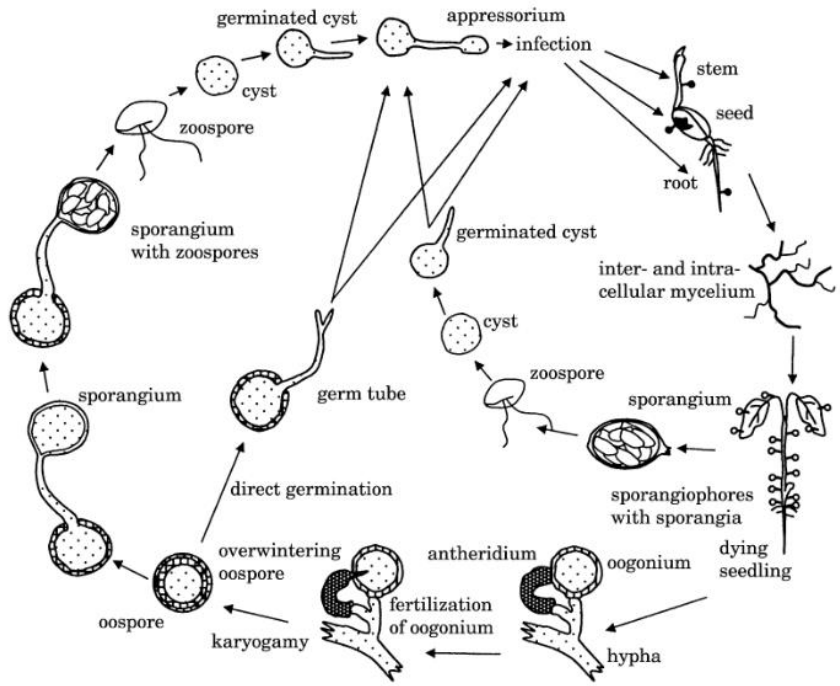

Figure 1.1: Life cycle of *Pythium* spp. (West *et al.,* 2003).

A white, rapidly growing mycelium is produced by *Pythium*. The mycelium plays an important role in the infection process as it produces sporangia which germinate by releasing germ tubes or vesicles. The germ tubes come into contact with the host plant, penetrating the host tissue to begin infection, and sometimes the germ tube produces another vesicle which releases secondary zoospores and this process may be repeated (Agrios, 2005).

Optimum conditions for infection are when the temperature is between 10-18°C; this is when germination by means of zoospores is induced. Zoospores need free water to be able to swim and infect plants. For survival, the pathogen lives in dead plant material as a saprophyte or a parasite of fibrous roots of plants. The pathogen may heavily colonise seeds or seedlings planted on infested soil. The severity of the disease is increased when the wetness of the soil is prolonged and the temperature is unfavourable for the growth of the host plant, e.g. when the temperature is too low for the host. When there is nitrogen surplus in the soil, and crop rotation is not practised disease severity increases (Agrios, 2005).

Figure 1.2: Rotten mesocotyl of maize seedlings infected with *Pythium* (Think Burrus, 2015).

1.2.1.2 *Rhizoctonia*

Rhizoctonia is a soil-borne basidiomycete responsible for major plant diseases that affect roots, stems, tubers, and other plant parts (Agrios, 2005). *Rhizoctonia* has a wide host range, including maize, potatoes (*Solanum tuberosum*, L.), cucumber (*Cucumis sativus* L.), and many cereals (Al-askar and Rashad 2015). One of the most common diseases caused by *Rhizoctonia* is damping-off and occurs on young

seedlings, which may be killed before or after emergence. Root lesions in seedlings and partly brown plants are also symptoms of *Rhizoctonia*. In cool, wet weather, reddish-brown lesions develop, and because of favourable environmental conditions, they grow in size and numbers affecting the roots and the whole base of the plant. Root infections by this pathogen may lead to yellowing, weakening, and sometimes death of the plant (Agrios, 2005).

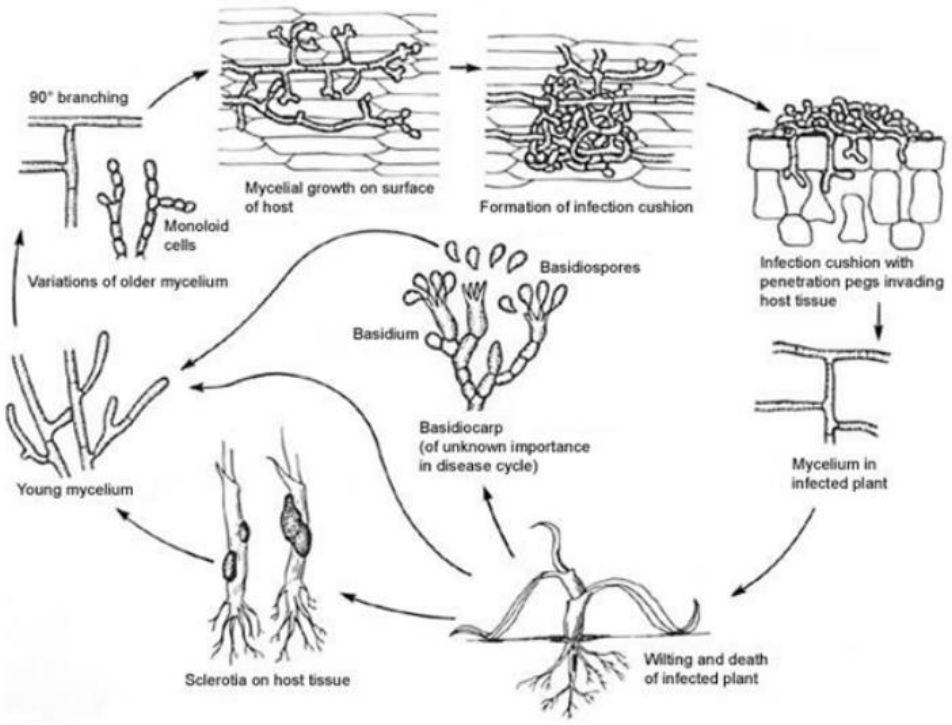

Figure 1.3: Life cycle of *Rhizoctonia solani* (Haque and Parvin., 2021).

In maize, *R. solani* may lead to reddish-brown lesions, which can be identified on the roots. Under favourable disease development conditions, seedlings may be wholly destroyed, or the pathogen impedes growth, while mature plants may lodge since they are not supported by strong roots (Sumner and Minton 1989). Infection of adult maize plants occurs at the pre-flowering stage, when plants are between 30-40 days after planting, and young plants can also be infected, leading to extreme blighting and destruction of the apical region. Diseased plants may seem bleached and soon become necrotic (Rani *et al*., 2013). In maize plants attacked by *R. solani*, the damage is governed by disease severity, how susceptible the host is, and disease favourable environmental conditions. When there are extended periods of rainfall, up to 100% of yield may be lost. When the rainfall is about 100mm, relative humidity varies between

90-100%, and plant densities are high at the pre-flowering stage, the development of banded leaf sheath blight (BLSB) is favoured (Hooda *et al*., 2015).

Like most fungal pathogens, *Rhizoctonia* overwinters in the soil as mycelium or propagative material and can also be carried in the seeds. Once the soil is infested with *Rhizoctonia*, the pathogen remains in the soil permanently. Dispersal of this pathogen is through rain, irrigation, and contaminated planting tools, vegetative and propagative material. This fungus thrives in moderately wet soils, while waterlogged or dry soils are not ideal for infection. The most favourable temperatures for infection are 15-18°C; even though infection usually occurs between these temperatures, there are races of *Rhizoctonia* tolerant to extremely high temperatures of up to 35°C. When conditions are not favourable for the host plant, for example when plant growth is slow due to unfavourable environmental conditions, infection of young plants is then accelerated (Agrios, 2005).

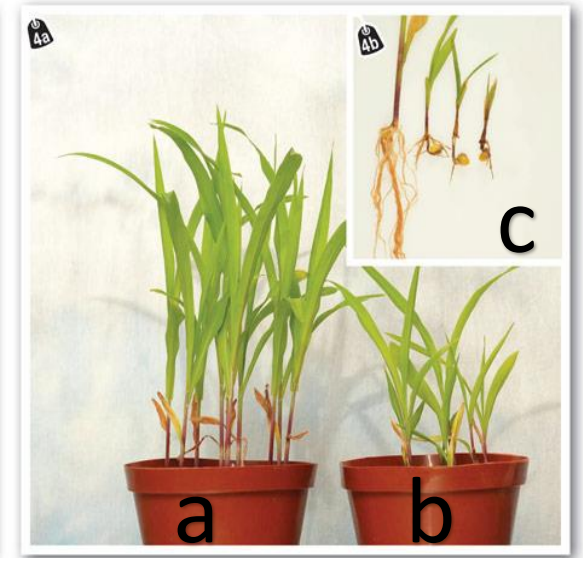

Figure 1.4: *R. solani* on maize seedlings a) Control with no pathogen, b) infected seedlings, stunting, c) control (left), *R. solani* root rot on maize seedlings (right), (Lamprecht, *et al*., 2013).

1.2.2 Economic importance

1.2.2.1 Pythium

When seeds or seedlings are infected with damping-off, a lower germination percentage as a result of the injury to the hypocotyl and mesocotyl can be observed, leading to a slow growth rate, seed, and root rots. Therefore, *Pythium* infections on crops may lead to substantial yield losses (Matthiesen *et al*., 2016). Various plant

pathogens cause root rots; however, the amount of yield loss due to these pathogens is dependent on the susceptibility of the host and also environmental conditions on which the infection process occurs (Tunali *et al*., 2008). About 30% of yield losses on cowpea (*Vigna unguiculata* L.) due to root rot fungi have been recorded (Suleiman and Emua, 2009).

A range of *Pythium* species is associated with yield losses thereby reducing bean (*Phaseolus vulgaris* L.) production in Western Kenya and Rwanda. These species include *P. echiulatum*, *P. oligandum*, P. *irregulare*, and *P. ultimum*. Approximately 70% of the bean production in Kenya and Rwanda has been lost due to *Pythium* root rots. *Pythium* species are the most common pathogens causing bean root rot in some African countries; because of this, between 1991 and 1993, farmers in Western Kenya and Rwanda stopped producing beans leading to starvation in these countries (Nzungize *et al*., 2012). According to Matthiesen *et al.*, (2016), about 3.7 million kg of maize yield have been lost due to *Pythium* spp. during 2012 and 5.8 million kg in 2013 in Canada and United States.

1.2.2.2 *Rhizoctonia*

Compared to other species, *R. solani* is the most common species causing disease on maize fields. The pathogen is destructive to plant parts such as,- the hypocotyls, seeds, and roots which are the underground parts of the plant (Da Silva *et al*., 2017). *R. solani* is of economic importance in several countries since it can cause yield losses of up to 100% (Hooda *et al*., 2015). Different groups of *R. solani* exist; these groups are separated according to their hyphal anastomosis and cultural characteristics into anastomosis groups (AG). A number of *R. solani* AG and subgroups are virulent towards maize, e.g. AG 2-2, AG-4 and AG 2-2 IIIB (Da Silva *et al*., 2017).

R. solani AG 2-2 destroys the crown and brace roots of maize, leading to considerable amounts of yield loss. This is because these roots are essential to increase the production of forage and grains (Sumner and Minton, 1989). In an experiment conducted by Sumner and Minton (1989), 40-50% of maize yield loss resulted from root rot from *R. solani*. This loss was higher than yield loss induced by nematodes. Da Silva *et al*., (2017), tested the effect of different seed treatments on root rots of maize. It was discovered that *R. solani* significantly reduced yield due to the lowering of root

length, volume, surface area, and the number of root tips. All these factors contribute remarkably to yield.

1.2.3 Management of *Pythium* and *Rhizoctonia* root-rot diseases.

i. Cultural practices

One of the most important strategies in reducing root rots is ensuring that there is a sufficient amount of water available to the host's root system (Naseri and Moradi, 2015). In order to successfully manage diseases caused by *Rhizoctonia,* wet, poorly drained soils should be avoided; one way of doing this is using sprinkler irrigation compared to flood and furrow irrigation which may lead to the accumulation of water, especially in low lying areas (Naseri and Moradi, 2015). Reduction of *Rhizoctonia* root rots can also be achieved by encouraging environmental conditions that are unfavourable for disease development and thus promote host plant growth (Naseri and Moradi, 2015). To control both *Pythium* and *Rhizoctonia* root rots, disease-free seeds should be adopted, and retained seeds should be avoided. Producers should use raised seedbeds in environmental conditions that promote rapid seedling growth. Wide in-row spacing allows good aeration of the soil surface and plants. Sterilisation of the soil with chemicals and crop rotation reduces disease incidence. Some cultural practices like good soil drainage, good air circulation, planting when environmental conditions are favourable for fast plant growth, and applying adequate amounts of nitrate forms of nitrogen have reduced disease incidence and severity of *Pythium* root rot (Agrios, 2005).

ii. Chemical control

The use of fungicides as seed or bulb treatments is the most used strategy to prevent the occurrence of root rot. Maize seed treatment by fungicides is the most adapted method of controlling root rot caused by *R. solani* so far (Al-askar and Rashad, 2015).

Fungicides such as, phenyl amides, mefenoxam, metalaxyl and Quinone outside inhibitors (QoIs) have been used as seed treatments in maize production to reduce the incidence of diseases caused by *Pythium* and other soil-borne pathogens (Matthiesen *et al*., 2016). Systemic fungicides combined with broad-spectrum fungicides is one of the most effective ways to reduce damping-off, seedling blights, and root rots caused by *Pythium, Rhizoctonia* and *Phytophthora* species. When the soil infestation levels are extremely high, or soil wetness is prolonged, seed treatments

followed by spraying of seedlings with the same or different fungicides is known to reduce disease severity during the early stages of plant growth (Agrios, 2005). The use of systemic fungicides and soil fumigants forms a major part of the management of *Rhizoctonia* root rot, even though, *R. solani* AGs and subgroups show various reactions towards different fungicides and fumigants (Singh *et al*., 2019).

iii. Biological control

In order to obtain optimum yields with good quality products, farmers utilise chemicals to reduce or prevent damages as a result of plant pathogens (Zheng *et al*., 2017). Even though chemical control of plant diseases has been used for years and has proven successful in most cases, recently focus has shifted to biological control strategies. Chemicals are regarded as a threat to natural biodiversity and human health, they influence the structures and functions of microorganisms in bulk soil and rhizosphere soil. When fungicides are used, there is always possibility of fungicide resistance (Hassan, 2017). For example, even though phenylamides and QoIs are being used as chemical control of soil-borne pathogens such as *Pythium,* they are considered as high risk for the development of fungicide resistance (Matthiesen *et al*., 2016). In order to avoid the side effects associated with the use of chemical control, biological disease management strategies have been developed (Hassan, 2017).

Biological control strategies are based on the use of other microorganisms to control plant diseases. Nzungize *et al*., (2012) suggested that microorganisms have the ability to protect common bean (*Phaseolus vulgaris* L.) against *Pythium* species through the production of antifungal metabolites, competition with the host for nutrients, niche exclusion, and parasitism of the pathogen or through induced plant resistance. Since soil-borne pathogens share a habitat with other microorganisms in a vigorous environment at the rhizosphere interface, biological control of these pathogens is quite complex (Nzungize *et al*., 2012). The rhizosphere represents enormous microbial activity that includes a high population of microorganisms, changes in pH, salt concentrations, osmotic and water potential making it difficult to control soil-borne pathogens (Nzungize *et al*., 2012).

To avoid chemical use, several microorganisms such as fungi, soil mycobacteria and mycophage nematodes can be used to reduce disease severity and incidence (Agrios, 2005). For example, cucumber seeds treated with *Pseudomonas putida* bacteria or

mycoparasite *Verticillium lecanii*, induces the production of phytoalexins and other host plant defence reactions (Agrios, 2005).

1.4 Endophytes

Amongst other biological control strategies, interest has been growing on the use of endophytes in plant disease management. Endophytes can be defined as microorganisms that are able to live asymptomatically within their host tissues and each plant species is regarded as a host to at least one endophyte (Jose and Christy, 2013). The ability of endophytes to live asymptomatically in host tissues may be because they biosynthesize the same chemical compounds as their host plant, as a form of survival in the host tissues (Martinez-klimova *et al*., 2017). Since endophytes inhabit the host plant with no evident symptoms, they have the ability to inhibit pathogen growth through competition (Hassan, 2017), and by releasing metabolites that induce host plant defence mechanisms (Martinez-klimova *et al*., 2017). It is likely that endophytes may serve as a source for new natural products that can be utilized in medicinal, agricultural and industrial uses (Huang *et* al., 2007).

Endophytes, especially those extracted from medicinal plants have gained interest as possible biocontrol agents in agriculture (Martinez-klimova *et al*., 2017). Wild species of crops may also provide endophytic microorganisms that inhibit the growth of plant pathogens (Abdallah *et al*., 2016). From studies conducted in the past, endophytes are suspected of displaying tissue specificity; for example it has been found that endophytic species are found in certain tissues of the host but not in other tissues and that the rhizosphere constitutes more endophytes than the aboveground part of the host plant (Zheng *et al*., 2017).The abundance of endophytes in the rhizosphere is probably due to the possibility of soil-borne microorganisms being more widespread and diversified than those found in the aboveground parts of the plant (Zheng *et al*., 2017).

1.4.1 Interaction between endophytes and plant pathogens

It has been suggested that the role endophytes play in plant disease management varies with host and endophyte species and also the relationship amongst the two (Bromfield *et al*., 2018). Researchers have suggested that endophytes have the ability to lower plant disease severity and have therefore gained preference over chemical use (Martinez-klimova *et al*., 2017). The mechanisms involved when endophytes act

as biocontrol agents (BCAs) include disturbing the colonization process of the pathogen to prevent initiation of disease through quorum sensing. Since endophytes are able to increase root volume produced by host plants, the plant can overpower cell death induced by pathogens (Martinez-klimova *et al*., 2017).

Endophytes harbour structurally distinct bioactive natural products, such as alkaloids, phenolic acids, flavonoids, quinones etc. which may be involved in inhibiting disease development (Huang *et al.*, 2007). Antibiotics, antiviral and insecticidal products have been reported from endophytes (Huang *et al.*, 2007) and secretion of hydrolytic enzymes and antibiotics is one of the traits that can be exploited in endophytes to impede colonization of host tissues by pathogens, insects or nematodes (Martinez-klimova *et al*., 2017). It has been suggested that bacterial endophytes slow down disease development by producing siderophores which induce the plant defence system and producing inhibitory compounds such as chitinases, diffusible antibiotics and volatile inhibitory compounds (Wicaksono *et al*., 2017).

1.4.2 Management of plant diseases using endophytes

Endophytes have been studied as potential BCAs against various plant pathogens. Table 1.2 represents published data on the use of endophytes as antagonist in plant disease management.

Table 1.2. Endophytes with antagonistic activities against plant pathogens

Pathogen	Disease	Antagonistic Endophyte	Host plant	Reference
1. *Alternaria panax*, *Fusarium solani, Phoma herbarum*	Root rot	*-Cladosporum oxysporum -Trichoderma koningiopsis*	*Panax notoginseng*	(Zheng *et al*., 2017)
2. *Phytophthora capsici*	Root rot	*Nigrospora sphaerica*	*Cornus florida*	(Mmbaga *et al*., 2018)
3. *P. capsici*	Root rot	*Pseudomonas aeruginosa P. putida B. megaterium*	*Piper nigrum* L.	(Aravind *et al*., 2009)
4. *F. solani, Verticillium dahliae*	Root rot Verticillium wilt	*F. proliferatum*	*Camptotheca acuminate*	(Ding *et al*., 2013)
5. *Sclerotinia sclerotiorum*	Head rot	*Macrophomina phaseolina*	*Ocumum sanctum*	(Chowdhary and Kaushik, 2015)
6. *S. sclerotiorum*	White mold	*Chaetomium globosum*	*Withania somnifera*	(Kumar *et al*., 2013)
7. *Cladosporium sphaerospermum*	Leaf spot	*Phomopis cassia*	*Cassia spectabilis*	(Gao *et al*., 2010)
8. *Ralstonia solanacearum*	Vascular wilt	*Streptomyces virginiae*	*Solonam lypersicum*	(Eljounaidi *et al*., 2016)
9. *F. oxysprum F. verticilloides, F. pallidoroseum and C. herbarum*	Maize Wilt	*Alternaria alternata Trichoderma koningii*	Maize	(Orole and Adejumo, 2009)
10. *F. solani*	Root rot	*Bacillus cereus*	*Cicer aritenum* L.	(Egamberdieva *et al*., 2017)

1.5 Medicinal plants as hosts for endophytes

For years, medicinal plants have been used for human well-being and as an important reserve for pharmaceuticals (Palanichamy *et al*., 2018). Therefore, it has been suggested that endophytes found in medicinal plants may produce natural bioactive products (Liu *et al*., 2016). Medicinal plants can control diseases caused by various microorganisms; hence they are used as substitutes of antibiotics (Khan *et al*., 2018). According to the world health organisation (WHO), approximately 80% of the world's population relies on traditional medicinal plants for their basic primary health care necessities (Khan *et al*., 2018). The reason for the ability of medicinal plants to cure human diseases may be the result of microorganisms that dwell within these plants (Wicaksono *et al*., 2017). Since they are regarded as a repository of endophytes (Huang *et al.*, 2007), medicinal plants may be used as novel antimicrobial substances (Khan *et al*., 2018).

Antifungal activity and cytotoxicity for some fungal endophytes have been reported against human pathogens (Egan *et al*., 2016). *Houttuynia cordata* Thunb. is a medicinal plant used in Chinese traditional medicine to cure inflammation, bronchitis infections of the upper respiratory cavity, cough, and other common human diseases. This plant has been reported to have antifungal, antiviral, anti-oxidative traits and is also able to increase immunity (Pan *et al*., 2016). A fungal endophyte identified as *Chaetomium globosum* EF18 was isolated from *Withania somnifera* and showed antifungal activity against *Sclerotinia sclerotiorum, F. oxysporum,* and *R. solani* (Kumar *et al*., 2013). Bacterial and fungal endophytes have also been isolated from *Teucrium polium* L., a medicinal plant that has been reported to have antioxidant, anticancer, antifungal and antibacterial activities for humans. The isolated endophytes were characterized as plant growth-promoting endophytes (Hassan, 2017).

1.5.1 *Arctotis arctotoides* (L.f) O. Hoffm

Arctotis arctotoides (L.f) O. Hoffm, belonging to the Asteraceae family is a common herbaceous decumbent medicinal plant in the Eastern Cape, South Africa. The plant grows as a roadside weed in coastal and summer rainfall areas of South Africa. Amongst the Xhosa people of the Eastern Cape, it is known as Ubushwa and as the African daisy in English (Afolayan, 2003; Afolayan *et al., 2007*; Badmus and Afolayan

2012). The plant grows up to 55-60cm in height at maturity, and the aerial part of the plant is usually covered with white hairy structures, which possibly secrete the plants' secondary metabolites (Badmus and Afolayan, 2012). According to the indigenous people of the Eastern Cape, extracts from *A. arctotoides* are used for the treatment of diseases such as epilepsy, indigestion, catarrh of the stomach, ringworm, wounds, pimples and insect bites (Afolayan, 2003; Afolayan *et al*., 2007; Badmus and Afolayan, 2012; Dlova and Ollengo, 2018).

Figure 1.5: *Arctotis arctotoides* (Picture taken by N. Yekelo, 2021).

Indigenous people use traditional medicinal plants in different forms, for example, they can be used as paste or decoction. In the Eastern Cape, the juice from leaves of *A. arctotoides* is applied as a topical paste on wounds (Afolayan *et al.,* 2002; Afolayan *et al*. 2007). Decoctions are also a common practice in traditional medicine, the decoction is prepared by cold or hot water, and concerning *A. arctotoides*, people wash with it daily (Afolayan *et al*., 2002; Dlova and Ollengo 2018).

Since extracts from the *A. arctotoides* are able to cure human diseases, research has been conducted to study the antimicrobial activity of compounds isolated from the plant. It has been shown that root and shoots extracts can inhibit the growth of some

bacterial and fungal human pathogens e.g. *Cladosporium herbarum* which causes asthma attack (Afolayan, 2003). Some research shows that extracts from *A. arctotoides* have antifungal activity against the growth of *Alternaria alternata*, *Aspergillus niger* (Afolayan *et al*., 2002), *A. flavus* and *Penicillium digitatum* (Afolayan, 2003). Extracts from the shoots were also able to inhibit the growth of bacterial pathogens, with inhibition of Gram-positive bacteria being higher than that of Gram-negative bacteria and some of the Gram-positive bacteria inhibited by the extracts were *Bacillus cereus* and *Staphylococcus aureus* (Afolayan, 2003).

Even though there is information on *A. arctotoides* and the compounds found in the plant which are responsible for the treatment of human diseases, no information is available on the use of extracts from this plant for plant disease management. Therefore, this study focuses on isolating endophytes from *A. arctotoides* and testing their antifungal activity against *Pythium* and *Rhizoctonia* root diseases of maize.

1.6 Conclusion

Biological control strategies are needed for plant disease management since fungicides have been associated with negative impacts on the environment, soil microbiome, and human beings.

Although research on the antagonistic activity of organisms towards plant pathogens exists, research on the use of endophytes for plant disease management is limited. Most researchers have isolated endophytes and tested them against pathogens of the endophyte host, meaning little information is available on the use of endophytes from a different host for controlling plant diseases on another host. Even endophytes isolated from medicinal plants have been used to control plant diseases of medicinal plants (Zheng *et al*., 2017).

Therefore, there is a high potential that endophytes, especially those isolated from medicinal plants, show antifungal activity towards plant pathogens and can also be formulated into antibiotics of plant diseases to reduce the use of fungicides.

The interaction between endophytes, pathogens, hosts and the environment they occupy influences the type of relationship seen on the host. Therefore, some

microorganisms may be pathogenic to other plants while they are non-pathogenic and reside as endophytes on other plants.

Published information on traditional medicinal plants and their use is limited; therefore most information is obtained through interacting with indigenous people. Traditional medicinal plants may be harbour compounds that are antagonistic towards plant pathogens just like they are to pathogens infecting humans.

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CHAPTER 2: Isolation, - and *in vitro* screening of bacterial endophytes from *Arctotis arctotoides* (L.f.) O. Hoffm against two root-rot pathogens, *Pythium* spp*.* and *Rhizoctonia solani*.

Abstract

Bacterial endophytes have been reported to produce antimicrobial compounds against fungal pathogens and metabolites that induce crop self defence mechanisms. In this study the medicinal plant, *A. arctotoides* was selected to screen for endophytes as potential antagonists against fungal root pathogens of maize. A total of 26 bacterial endophytes were isolated from the leaves, stem and roots of *A. arctotoides* and screened *in vitro* for their antagonistic activity against two root rot pathogens *Pythium* spp. and *R. solani,* using dual culture assays. Out of the 26 isolates, 10 were antagonistic against *R. solani* and 11 were antagonistic against *Pythium* spp. These endophytes caused a significant ($P \leq 0.05$) reduction in pathogen mycelial growth during *in vitro* studies. The percentage inhibition ranged from 17 - 50% and 8 - 64% for *R. solani* and *Pythium* spp., respectively. Using Internal Transcribed Spacers (ITS) sequencing, the best isolates against *R. solani* were identified *as Proteus vulgaris* NYR13, *Lysinibacillus pakistanensis* NYL21, *Bacillus cereus* NYR11, *Morganella morganii* KC-Tt-01 NYL20, *P. mirabilis* NYR9, *M. morganii* L143 NYR3, *Stenotrophomonas maltophilia* NYL15, *Pseudomonas putida* NYR14, *M. morganii* DG56-16 NYS3 and *Bacillus* spp. NYR2. Those inhibitory against *Pythium* spp. were identified as *Serratia marcescens* NYS8, *M. morganii* AR_0133 NYL12, *M. morganii* L143 NYR3, *Alcaligenes faecalis* NYS7, *Ralstonia* spp. NYR8, *Bacillus* spp. NYS9, *B. cereus* NYR11, *Myroides odoratus* 6G NYL18, *P. putida* NYL16 and *M. morganii* OG003 NYL14. The endophytes with inhibitory effects have the potential to be used as biological control agents against *Pythium* spp. and *R. solani* root rot of maize and were selected for further evaluation under greenhouse conditions.

Keywords: *A. arctotoides*, antagonism, biological control, root rot pathogens.

2.1 Introduction

Rhizoctonia and *Pythium* species are among the numerous fungal pathogens that cause various diseases on cereals, grasses, vegetable crops, ornamental plants, fruit and forest trees. *Pythium* diseases are mostly common in seedlings and young plant tissues where they cause damping-off, root rot, wilt and lodging (Schmidt *et al.*, 2020). Even though *Pythium* spp*.* is common in young plants, it has also been reported on root tips of older plants, stems and foliage of some grasses resulting in stunting, lodging and heads of maize (*Zea mays* L.) hanging downwards. All these disease symptoms lead to huge yield losses of up to 100% (Al-sadi *et al.*, 2012; Mitsuhashi *et al.*, 2015). In some countries, production of certain crops has been limited due to root rot caused by *Pythium* spp*.* (Mitsuhashi *et al.*, 2015).

R. solani is the most common species of *Rhizoctonia*. It causes damping-off and root rot diseases of many crops, worldwide. The pathogen infects seeds, roots, and hypocotyls (Da Silva *et al*., 2017). Maize plants infected with *R. solani* develop reddish-brown to black lesions on the crown and brace roots. Root rot caused by this pathogen may lead to lodging of plants (Fähler and Peterson, 2004). *R. solani* is well known in the cropping world, as it can cause up to 30% yield losses in maize (Fähler and Peterson, 2004). The pathogen is necrotrophic, therefore it survives by killing plant parts and those dead plant parts become a nutritional source to the pathogen (Wang and Zhuang, 2019).

Cultural practices such as raised seed beds, crop rotation, good soil drainage and use of disease-free seeds have been used to control both *Pythium* spp. and *R. solani* root rot diseases. Other methods include chemical control which is the most regularly used strategy for management of root diseases. Metalaxyl is the most used fungicide for controlling *Pythium* spp. whereas *R. solani* is mostly controlled with broad spectrum fungicides such as, azoxystrobin, fludioxonil or thiabendazole (Da Silva *et al.,*2017). Biological control of plant diseases is another method that is used as an alternative to chemical control because chemical control methods have been reported to be hazardous to the environment and human health (Abbas *et al*., 2019).

The use of endophytes as biological control agents against fungal plant pathogens is based on the premise that they release metabolites that induce host plant defence mechanisms and inhibit pathogen growth through competition (Martinez-Klimova *et*

al., 2017). Endophytic *Trichoderma harzanium* and *T. lentiforme* were isolated from healthy watermelon (*Citrullus lanatus* [Thunb.]) and showed antagonistic activity against collapse (*Monosporascus cannonballus* Pollack and Uecker*)* of watermelon in dual culture assays (Gonzalez *et al*.., 2020). It has been shown that mulberry (*Monis alba* L.) is a host to the endophyte *Bacillus amyloliquefaciens* ZJU1 which induces resistance to *Botrytis cinerea* Persoon. of mulberry (Xie *et al*., 2020). As *A. arctotoides* has shown to have bioactivity against various human pathogens (Dlova and Ollengo., 2018), this study evaluates the potential of endophytes isolated from *A. arctotoides* as antagonists against fungal plant pathogens.

Therefore, the objectives of this study, was to isolate bacterial endophytes from *A. arctotoides* and evaluate their antagonistic activity against *Pythium* spp. and *R. solani* root rot pathogens of maize.

2.2 Materials and methods

2.2.1 Isolation of bacterial endophytes

For the isolation of endophytes, *A. arctotoides* plant samples were collected from different areas in the Eastern Cape, Republic of South Africa. This was to see the diverse microbial populations under various climatic conditions. The plants were uprooted and placed in paper bags with soil from the rhizosphere. Once in the paper bag, the plants were sprinkled with water to prevent immediate wilting and transported to the laboratory. Isolation was done within 24 hours after uprooting the plant. Procedure used for the isolation of endophytes was the method described by Zheng *et al.*, (2017) with some modifications. Plants were thoroughly washed under running tap water to remove soil particles. After that, the plant samples were cut and separated into different plant parts, namely, leaves, stem and roots. The different plant parts were then separately rinsed three times with sterile distilled water. They were then dipped in 99% ethanol for one minute and rinsed three times with sterile distilled water. Plant parts were placed on sterile filter papers and allowed to air dry on a laminar flow bench.

After air drying, bacterial endophytes were isolated by cutting the samples into approximately 1cm pieces and placed in the middle of 90mm petri dishes with Nutrient Agar (NA) and Tryptone Soy Agar (TSA) respectively. The plates were then incubated for seven days at 25°C and pure cultures of distinct bacterial colonies were selected according to colony colour, shape, size and elevation. These colonies were then

streaked onto new NA and TSA agar plates to obtain single colonies. Different isolates were then stored in 20% (w/v) glycerol stocks at -80°C.

2.2.2 Gram staining of isolated bacterial endophytes

The Gram stain procedure was conducted using the method described by Eze *et al.,* (2010) with some modifications. A drop of sterile distilled water was placed on a microscope slide. Using a sterile inoculating loop, a single colony of bacterial isolate was taken from the agar plate and smeared in the drop of water. The smear was then left to air dry on the laminar flow bench and heat-fixed by passing over a Bunsen burner flame three times. The heat fixed smear was then flooded with crystal violet solution for one minute and then rinsed with distilled water. Iodine, which served as a mordant was then added and also allowed to stand for one minute, and rinsed with distilled water. Ninety nine percent (99%) ethanol was added as a decolourizer and rinsed immediately with distilled water. Safranin was added as a counter stain and allowed to stand for one minute before being rinsed with distilled water. The slides were then allowed to air dry in the laminar flow bench before observing under the microscope at 100X magnification under glycerol.

2.2.3 Pathogen isolation (*Pythium* spp.) and sourcing (*R. solani*).

Pythium was isolated using the baiting method (Ferguson and Jeffers, 1999). One litre of sterile distilled water was added to 500g of soil and incubated in the dark at room temperature overnight. Carrots (*Daucus carota subsp. sativus*) were surface sterilized with 99% ethanol for 1 minute and cut approximately into 1cm squares. The carrot pieces were then floated on the soil-water suspension and incubated at 25°C for four days. Carrot baits were rinsed with autoclaved distilled water, air dried on sterile filter papers on the laminar flow bench. They were then transferred to water agar (WA) and incubated at 25°C for seven days. White mycelium growing out of the carrot pieces was then transferred to new WA plates and incubated for seven more days at 25°C. The mycelium was then evaluated under light microscope, the hyphae were not dichotomously branched. There were swellings on the hyphae that were considered as oogonia, characteristic to *Pythium* spp.

The *R. solani* culture used in this study was obtained from the fungal culture collection at the Discipline of Plant Pathology, University of KwaZulu-Natal, Pietermaritzburg, Republic of South Africa.

2.2.4 Pathogenicity test of *Pythium* spp.

The pathogenicity of the *Pythium* isolate was done under greenhouse conditions. Untreated maize seeds (cultivar DKC 93-74BRGEr1) obtained from Monsanto (Pty) Ltd, Sandton, Republic of South Africa, were surface sterilized with distilled water and allowed to air dry on the laminar flow bench overnight. Three pots (500ml volume) were filled with topsoil and one seed planted in the middle of each pot. The pathogen was inoculated into the soil approximately 3cm away from the seed using four agar plugs (4x4 mm²) carrying the mycelium of the pathogen. There were three control pots which were not inoculated with the pathogen. At plant, each pot was supplemented with 3g NPK fertiliser 2:3:4 (30) plus 0.5% Zn from Omnia Nutriology, Bryanston, South Africa. After germination, the plants were allowed to grow for 21 days and were irrigated daily with 50ml of tap water every day.

At the end of the growth period, root rot symptoms such as, brown root lesions, shorter root length were compared to the uninoculated control.

2.2.5 Storage of bacterial isolates (endophytes) and pathogens

Bacterial endophytes with different characteristics in terms of colony shape, colour and elevation were each stored in 1.5ml Eppendorf tubes with sterile 20% (w/v) glycerol. There were five replicates per each isolate. Sterile inoculating loop was used to pick a single bacterial colony from the agar plate and the colony gently released in the glycerol in the Eppendorf tubes and hand shaken to form a homogenous mixture. Thereafter, the tubes were stored in an ultra-low freezer at -80°C.

The pathogens, *Pythium* spp. and *R. solani* were separately stored on barley seeds (Kidane, 2008). The barley seeds were first soaked in distilled water overnight and then double-sterilized in an autoclave at 121°C for 1 hour before inoculating with agar plugs of the pathogens. Inoculated barley seeds were then incubated at 25°C for two weeks and later kept at ambient temperature until further use.

2.2.6 *In vitro* screening of the isolates

Antifungal activity of the 26 bacterial endophytes against *R. solani* and *Pythium* spp. was tested using dual culture assays on 90mm PDA plates (Wicaksono *et al.*, 2017). Antagonistic activity of bacterial endophytes, was determined by placing 4x4mm² agar plug with the mycelium of the pathogen in the middle of the agar plate. A loop-full of the endophyte was inoculated 30mm away from the pathogen on four sides around

the pathogen. For the control, the pathogen was inoculated in middle of the agar plate without any bacterial endophyte inoculations. The bioassay plates including the control were incubated at 25°C until the control plate was completely colonised. When the pathogen in the control had colonised the whole plate, inhibition percentage of the endophytes were determined. There were three replications for each bacterial and pathogen interaction. The inhibitory effect of each endophyte was calculated using the formula by Landum *et al.*, (2016) as follows:

$$Inhibition\ (\%) = \left(\frac{R1 - R2}{R1}\right) X\ 100$$

Where R1 is the radial hyphal growth in the control plate and R2 is the radial hyphal growth in the test plate.

2.2.7 Identification of bacterial antagonists

The best bacterial endophytes antagonistic towards *R. solani* and *Pythium* spp. were selected based on their percentage inhibition. These were sent to Inqaba Biotechnological Industries (Pty) Ltd (Pretoria, RSA), for Internal Transcribed Spacers (ITS) sequencing and molecular identification to a species level. The identities were obtained using the NCBI Basic Alignment Search Tool (BLAST).

2.2.8 Data analysis

The percentage inhibition of the endophytes towards the two pathogens was analysed statistically using Analysis of Variance (ANOVA GenStat 18th edition). Means were separated using Duncan's Multiple Range Test (DMRT) at P= 0.05. All experiments were repeated once.

2.3 Results

2.3.1 Isolation of bacterial endophytes

A total of 26 bacterial endophytes were isolated from *A. arctotoides*. These are presented in Table 2.1. The root of the plants had the lowest number of bacterial endophytes compared to the stem and the leaf as they both had the same number of bacterial endophytes. Thirty percent (30%) of the isolated bacterial endophytes were isolated from the root, with isolations from the leaf and stem making up the remaining 70% (35% of the bacterial endophytes each).

Table 2.1 Bacterial isolates from *A. arctotoides* - and their corresponding gram stain reactions.

Endophyte isolate	**Plant part**	**Gram stain reaction**	**Location**
NYS1	Stem	Negative	Zanyokwe
NYS2	Stem	Negative	Dohne ADI, Stutterheim
NYS3	Stem	Negative	Dohne ADI, Stutterheim
NYS4	Stem	Negative	N2 road Ezintabeni, Mthatha
NYS6	Stem	Negative	Lwaleni, Idutywa
NYS7	Stem	Negative	Bumbane, Idutywa
NYS8	Stem	Negative	Bumbane, Idutywa
NYS9	Stem	Positive	Bumbane, Idutywa
NYS11	Stem	Negative	Ncora, Cofimvaba
NYR2	Root	Negative	Zanyokwe
NYR3	Root	Negative	Dohne ADI, Stutterheim
NYR8	Root	Positive	Lwaleni, Idutywa
NYR9	Root	Negative	Lwaleni, Idutywa
NYR10	Root	Negative	Ngudwane, Idutywa
NYR11	Root	Positive	Ndakeni, Ntabankulu
NYR13	Root	Positive	Ndakeni, Ntabankulu
NYR14	Root	Negative	Ncora, Cofimvaba
NYL3	Leaf	Positive	N2 road Ezintabeni, Mthatha
NYL12	Leaf	Negative	Lwaleni, Idutywa
NYL14	Leaf	Negative	Ngudwane, Idutywa
NYL15	Leaf	Positive	N6 road, Stutterheim
NYL16	Leaf	Negative	Ndakeni, Ntabankulu
NYL17	Leaf	Positive	Ndakeni, Ntabankulu
NYL18	Leaf	Negative	Ndakeni, Ntabankulu
NYL20	Leaf	Positive	Ndakeni, Ntabankulu
NYL21	Leaf	Negative	Ncora, Cofimvaba

2.3.2 Gram staining of isolated endophytes

The results of the Gram stain procedure are presented in Table 2.1. The results indicated that 18 of the bacterial endophytes were Gram negative and 8 were Gram positive.

2.3.3 Pathogenicity test of the fungal pathogen

Two weeks after emergence, some of the plants started to show post-emergence damping-off and had yellow lower leaves. When the plants were uprooted, the inoculated roots had fewer root hairs and shorter plant height compared to the control. Plants inoculated with *Pythium* spp. had brown root lesions. The pathogen was re-isolated from the diseased plants. This was done by cutting the root lesions with a sterilised blade and placing it in the middle of petri dishes containing WA. These were then incubated at 25°C for seven days and the white mycelium was then cultured into new plates.

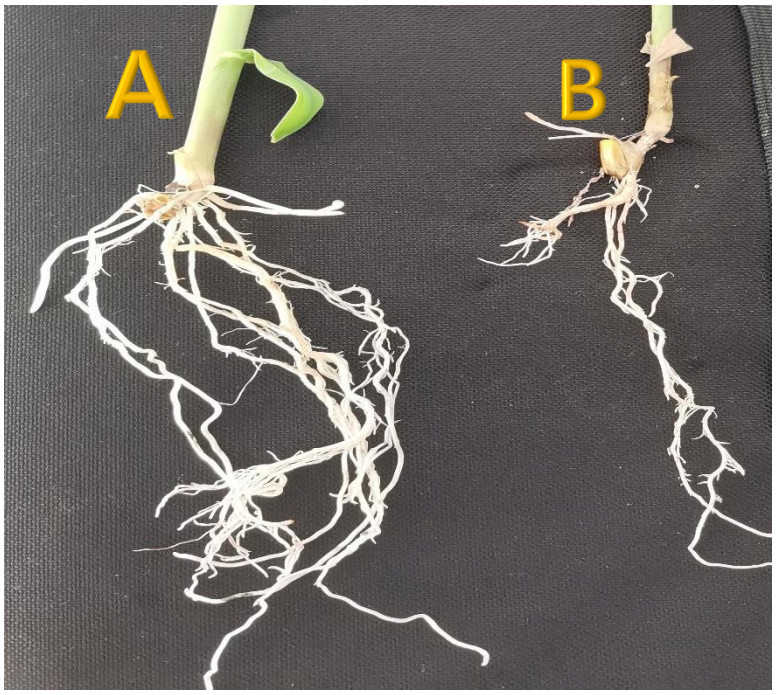

Figure 2.1: Infection of maize seedlings by *Pythium* spp. (A): control with no inoculation, (B) maize seedling infected with *Pythium* spp.

2.3.4 Dual culture bioassays

2.3.4.1 Rhizoctonia solani

Table 2.2 Mean percentage inhibition of the 10 bacterial endophytes with inhibitory effect against *R. solani* for experiments 1 and 2.

Endophyte isolates	**Mean % Inhibition (Exp. 1)**	**Mean % Inhibition (Exp. 2)**
NYS3	41.00bc	41.33ab
NYR2	30.67d	34.00cde
NYR3	23.00e	44.00a
NYR9	50.00a	40.00abc
NYR11	17.67e	28.33e
NYR13	35.33cd	37.67abcd
NYR14	45.00ab	41.67ab
NYL15	35.67cd	35.67bcd
NYL20	40.67bc	31.33de
NYL21	45.00ab	42.33ab
Control	0.00f	0.00f
P value	<.001	<.001
L.S.D	6.951	6.156
S.E.D	3.308	2.930
CV%	11.1	9.5

*Mean % inhibition of three replicates, values with the same letters within a column have no significant differences according to Duncan's multiple range test at 5% significance level.

All 26 endophytic bacterial isolates were tested against *R. solani*, and only 10 displayed an inhibitory effect. Table 2.2 represents the inhibition percentage of the bacterial endophytes against *R. solani*. Only five of the 26 bacterial endophytes (Table 2.2) had inhibition percentage greater than 40%. In Experiment 1, two of the five isolates were isolated from the roots (NYR9, NYR14), two from the leaves (NYL20, NYL21) and one from the stem (NYS3). As presented in Table 2.2, in Experiment 2,

three isolates were from the roots (NYR3, NYR9, and NYR14), one from the stem (NYS3) and one from the leaves (NYL21). The mean inhibition percentage of the endophytes against *R. solani* ranged between 17.67% (NYR11) and 50.00% (NYR9).

Bacterial endophyte NYR9 in Experiment 1 and NYL21 in Experiment 2, had a better inhibitory effect on *R. solani* as indicated in Table 2.2.

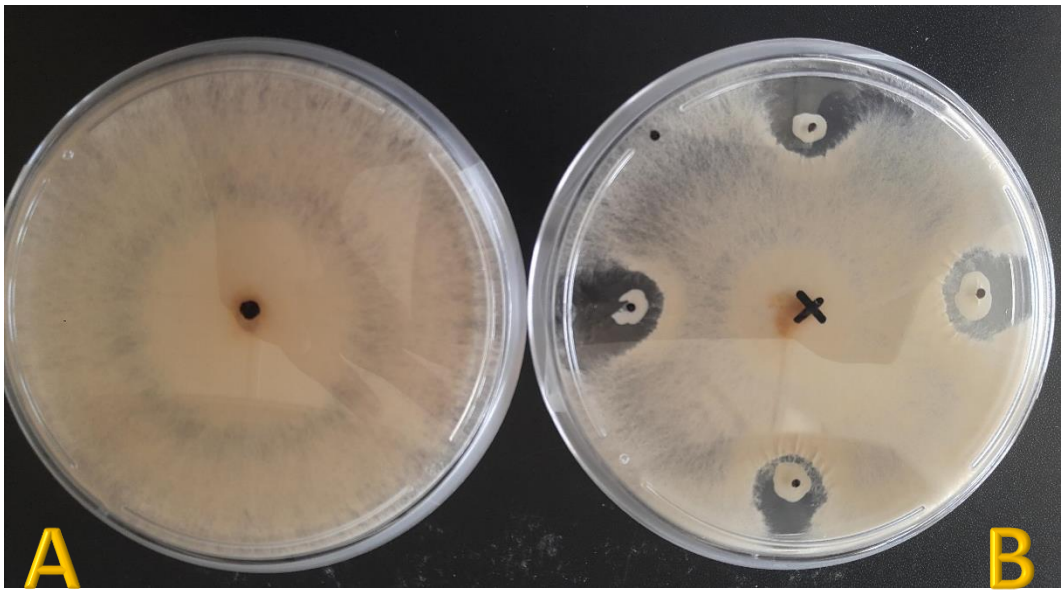

Figure 2.2: *In vitro* assay of NYR13 against *R. solani.* (A) Control plate with *R. solani* only. (B) Dual culture of NYR13 and *R. solani* showing visible zones of inhibition.

2.3.4.2 *Pythium* spp.

Table 2.3 represents the mean percentage inhibition of the bacterial endophytes against *Pythium* spp. A total of 11 endophytes showed antifungal activity against *Pythium* spp. in the dual culture assay. The endophytes significantly reduced the growth of the pathogen ($P < 0.001$). As shown in Table 2.3, NYS8 significantly inhibited *Pythium* spp. in both experiments compared to the other endophytes. Mean percentage inhibition towards *Pythium* spp. ranged from 8.33% (NYL20) to 64% (NYS8). Mean inhibition (%) for all the endophytes towards *Pythium* spp. was lower than 40% except for NYS8.

Table 2.3: Mean (%) inhibition of endophytes against *Pythium* spp. conducted *in vitro* over two separate experiments.

Endophyte	**Mean % Inhibition (Exp.1)**	**Mean % Inhibition (Exp. 2)**
NYS7	28.67bc	27.00b
NYS8	64.00a	52.67a
NYS9	21.33cd	16.33cd
NYR3	29.00bc	27.67b
NYR8	26.67bcd	28.33b
NYR11	20.00de	17.67cd
NYL12	31.67b	31.00b
NYL14	13.00ef	14.33de
NYL16	19.00def	24.00bc
NYL18	19.33def	15.67de
NYL20	11.67f	8.33e
Control	0g	0g
P value	<.001	<.001
L.S.D	7.366	7.012
S.E.D	3.531	3.361
CV%	16.7	17.2

*Mean inhibition (%) of three replicates for each treatment, means with the same letters have no significant differences between them according to Duncan's multiple range test at 5% significance level.

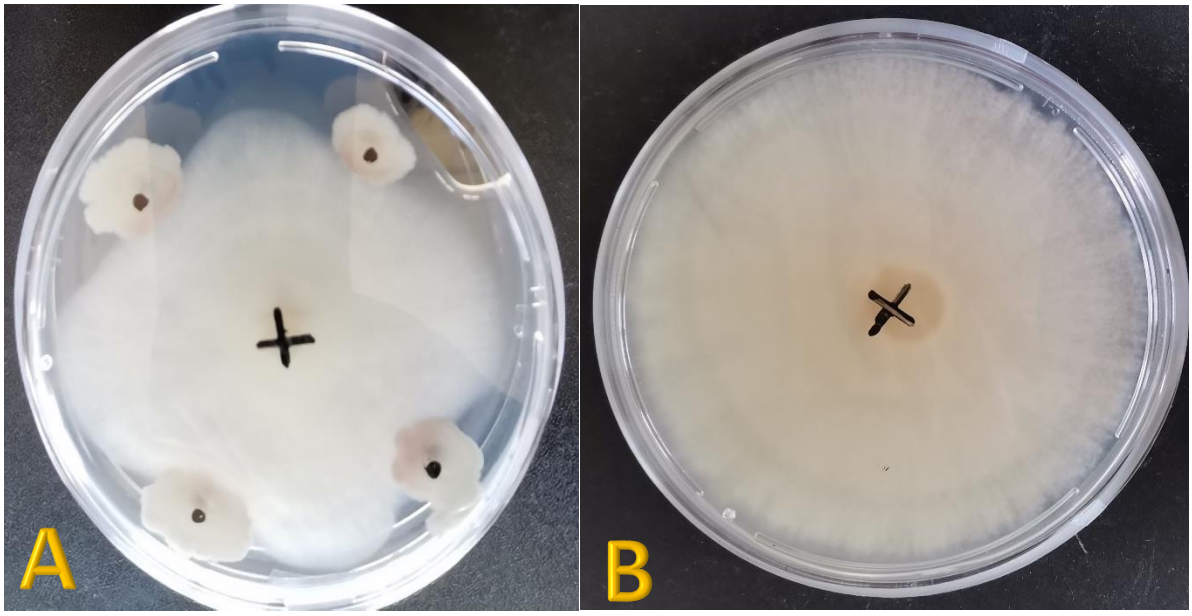

Figure 2.3: *In vitro* assay of NYS7 against *Pythium* spp. (A) Dual culture of NYS7 and *Pythium* spp. showing visible zones of inhibition.

2.3.5 Identification of bacterial isolates

Table 2.4 and 2.5 show the identities of the bacterial endophytes with inhibitory effects against *R. solani* and *Pythium* spp. *in vitro* according to the BLAST results.

Table 2.4 Identities of bacterial endophytes with inhibitory effects against *R. solani* root rot pathogen *in vitro* according to the BLAST results.

Isolate	**BLAST identification**
NYR2	*Bacillus* spp.
NYR3	*Morganella morganii* L143
NYR9	*Proteus mirabilis*
NYR11	*Bacillus cereus*
NYR13	*Proteus vulgaris*
NYR14	*Pseudomonas putida*
NYS3	*Morganella morganii* DG56-16
NYL15	*Stenotrophomonas maltophilia*
NYL20	*Morganella morganii* KC-Tt-01
NYL21	*Lysinibacillus pakistanensis*

Table 2.5 Identities of bacterial endophytes with inhibitory effects against *Pythium* spp. root rot pathogen *in vitro* according to the BLAST results.

Isolate	**BLAST identification**
NYR3	*Morganella morganii* L143
NYR8	*Ralstonia* spp.
NYR11	*Bacillus cereus*
NYS7	*Alcaligenes faecalis*
NYS8	*Serratia marcescens*
NYS9	*Bacillus* spp.
NYL12	*Morganella morganii* AR_0133
NYL14	*Morganella morganii* OG003
NYL16	*Pseudomonas putida*
NYL18	*Myroides odoratus* 6G
NYL20	*Morganella morganii* KC-Tt-01

2.4 Discussion

It has been reported that different endophytes can be isolated from plants of the same genus and species that grow in different regions or in the same region with different environmental conditions (Nair and Padmavathy, 2014). This observation is in agreement with the results in this study, as different endophytes were isolated from *A. arctotoides* collected from different or the same region/s.

Only three of the isolated endophytes (identified as *B. cereus* (NYR11), *M. morganii* L143 (NYR3) and *M. morganii* KC-Tt-01 (NYL20)) inhibited both *Pythium* and *R. solani*. *B. cereus* has been reported to have antagonistic effects against root rot pathogens such as *R. solani*, *Fusarium* spp. and *Macrophomina phaseolina in vitro* (Dawar *et al*., 2010). Endophytes that had the ability to inhibit or slow down the growth of the pathogens might have done so because they produce metabolites that inhibit growth of the pathogens (Fadiji and Babalola, 2020).

Sixty percent (60%) of the endophytes with inhibition against *R. solani* were isolated from the roots of *A. arctotoides*. The rhizosphere is a very hostile environment with a diverse microbial ecosystem that is highly competitive (Zheng *et al.*, 2017). It is

therefore likely that endophytes from the rhizosphere have high inhibitory effects compared to those isolated from other plant parts. However, only 27% of the endophytes with inhibitory effects against *Pythium* spp. were isolated from the roots. This percentage can be attributed to the genetic variations involved in the pathogen-endophyte interaction (Song *et al*., 2021). Forty-five percent (45%) of the endophytes with inhibitory effects against *Pythium* spp. were isolated from the leaves. Leaves have a larger surface area compared to other plant parts, hence, endophytes isolated from the leaves are diversified (Husseiny *et al.,* 2021). Previous research (Afolayan *et al.*, 2008), reports that indigenous people of the Eastern Cape Province in the Republic of South Africa use pastes and decoctions made from *A. arctotoides* leaves. Hence, endophytes from the leaves might be responsible for the metabolites that inhibit human and plant pathogens.

The Gram-positive *Bacillus* spp. have been studied extensively as potential BCAs of various plant pathogens (Saha *et al*., 2012). Saha *et al*., (2012), reported that *Bacillus subtilis* secretes antibiotics such as, iturins, surfactins and zwittermicin, including hydrolytic enzymes (protease, chitinase, lipase, amylase and glucanase). In this study three *Bacillus* isolates (*Bacillus* spp. (NYR2), *B. cereus* (NYR11) and *Bacillus* spp. (NYS9)) inhibited the growth of *Pythium* spp. and *R. solani in vitro.* Bacterial endophytes in the *Bacillus* genera, like those isolated in this study, are able to form endospores which help survive adverse conditions and increase shelve life as a BCA (Chen *et al*., 2020). The ability of the *Bacillus* endophytes isolated in this study to inhibit both pathogens might be attributed to these characteristics associated with *Bacillus* BCAs.

Bacillus spp. are some of the most promising microorganisms that can be used as BCAs against *Pythium* and *Rhizoctonia* root rots (Zhang *et al*., 2021). Only 19.23% of the bacterial isolates in this study belong to the genus *Bacillus*. In a study conducted by Abbas *et al*., (2019), *B. cereus* was able to reduce disease incidence and severity of tomato root rot caused by *R. solani*. This bacterial strain also inhibited mycelial growth of *R. solani in vitro*. The *B. cereus* (NYR11) strain identified from this study was one of the best 10 bacterial isolates with inhibitory effects against *R. solani* and *Pythium* spp.

Out of the 26 endophytic bacteria isolated in this study, 26.93% were identified as different strains of *M. morganii.* However, *M. morganii* is a human pathogen that causes wound and urinary tract infections (Liu *et* al., 2016). Similarly to plant pathogens, human pathogens are able to survive in plants and soil for extended periods. Human pathogens are able to live as endophytes in plants since they have the ability to attach, colonize plant surfaces and interact with other microbes in their host plant (Fletcher *et al*., 2013). Even though *M. morganii* was able to inhibit root rot pathogens in this study, using it as BCA might be harmful towards human beings. This is based on the idea that human pathogens stay within plant tissues and their virulence will be activated once consumed by humans (Fletcher *et al*., 2013). There is no available literature on the use or screening of *M. morganii* against the root rot pathogens used in this study. However, the potential of *M. morganii* to be used as BCAs has been reported against *Paenibacillus larvae* (Al-Ghamdi *et al*., 2019).

Pseudomonas protegens FD6 significantly reduced the growth of *R. solani* root rot pathogen *in vitro* (Zhang *et* al., 2021), therefore *Pseudomonas* spp. have potential to inhibit root rot pathogens. Two bacterial endophytes (NYR14 and NYL16) in this study were identified as *P. putida*. and NYR14 inhibited mycelial growth of *R. solani* while *P. putida* NYL16 inhibited *Pythium* spp. The bacterial endophyte *A. faecalis* (NYS7) reduced mycelial growth of *Pythium* spp. in this study. A bacterial strain (*A. faecalis* BHU M7) isolated from the medicinal plant, *Andrographis paniculata*, was reported to induce various defense mechanisms against the collar-rot pathogen (*Sclerotium* rolfsii) in okra (*Abelmoscus esculantus*) plants (Ray *et al*., 2016). Therefore, this correlates with the outcome of the bioassays done in this study, where the organism showed antimicrobial properties against *Pythium* spp.

Although antifungal activities of extracts from *A. arctotoides* have been studied (Afolayan., 2003, Otang-Mbeng *et al*., 2012), there is no available literature on isolation of endophytes from *A. arctotoides* and their potential antifungal effect on *R. solani* and *Pythium* spp. This study therefore contributes to limited information dealing with the isolation of endophytic bacteria as potential BCAs against *R. solani* and *Pythium* spp. The potential of these endophytes against the two pathogens will be tested under greenhouse conditions using maize as the host crop in Chapter Three.

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CHAPTER 3: Evaluation of selected bacterial endophytes against maize root rot pathogens, *R. solani* and *Pythium* spp. under greenhouse conditions.

Abstract

Maize root rot pathogens are prevalent throughout the world. Fungicides are the most common control strategies used against these pathogens, but alternative control strategies such as biological control are being pursued due to pathogen resistance issues. In this study, the potential of 18 bacterial endophytes previously screened *in vitro* against *R. solani* and *Pythium* spp. were evaluated on maize under greenhouse conditions. The endophytes were applied as a seed treatment and pathogen inoculated on the rhizosphere except in the control. The parameters measured were, plant height once a week for six weeks, root length, number of root lesions, root and shoot weight at harvesting. In relation to plant height, there were significant ($P<0.05$) differences between treatments in all experiments. Maize plants treated with the endophytes *Bacillus cereus* NYR11, *Proteus mirabilis* NYR9, and *Morganella morganii* DG56-16 NYS3 against *R. solani* and *Myroides odoratus* 6G NYL18, *Alcaligenes faecalis* NYS7, and *Ralstonia* spp. NYR8 against *Pythium* spp. showed low numbers of root lesions, increased root length, high root and shoot weights. These endophytes were regarded as the best treatments as they were among the top five treatments in most of the measured parameters. The performance of these isolates indicate that they have potential as biocontrol agents of two root rot pathogens on maize.

Keywords: Bacterial endophytes, biological control, root rot

3.1 Introduction

Maize (*Zea mays* L.) is an economically important crop worldwide and in sub-Saharan Africa (Kosemani and Bamgboye, 2021). It is regarded as a staple food, especially for smallholder farmers (Fischer and Hajdu, 2015). Maize is one of the most cultivated crops throughout the world because of its richness in nutrients such as, - fibre, carbohydrates, proteins and Vitamin A, C and E (Kosemani and Bamgboye, 2021). Farmers also use maize, mainly green maize forage, for livestock since it serves as an energy-rich feed (Kosemani and Bamgboye, 2021). With maize being produced on a large scale, root rot pathogens are largely distributed leading to reduced yield

(Galindo-Castaneda *et al*., 2019). *Pythium* spp. and *Rhizoctonia* spp. are among the four most common root rot pathogens, along with *Fusarium* spp. and *Phoma terrestis* (Galindo-Castaneda *et al*., 2019). Damping-off is usually associated with *Pythium* spp. and is mainly controlled by applying the metalaxyl fungicide as a seed treatment (Hinai, *et al.,* 2010). Maize plants infected with *R. solani* display symptoms of yellowing, damping-off, crown, and brace root rots (Kiprovski *et al*., 2012). The use of the fungicide penthiopyrad as a seed treatment effectively manages *R*. *solani* (Liu *et* al., 2021). Fungicides should be applied more than once to effectively control plant diseases, making it challenging to control root rot (Anand *et al*., 2009).

Consistent use of fungicides may lead to the pathogen developing resistance (Marian *et al*., 2018). Moreover, the disadvantage of using synthetic fungicides is that some are not eco-friendly and have side effects on human health (Fadiji and Babalola, 2020). Other than fungicide application, *Rhizoctonia* spp. and *Fusarium* spp. root rot pathogens can be managed by cultural practices such as, crop rotation and planting in well-drained soils (Naseri and Moradi, 2015). Biological control of plant diseases is an alternative option. It is the use of naturally occurring microorganisms as antagonists against plant pathogens. These microorganisms inhibit the growth of plant pathogens by releasing antibiotics, inducing systemic resistance, producing lytic enzymes and/or competing for resources in the host plant and in the rhizosphere (Abbas *et al*., 2019).

Numerous bacterial organisms belonging to the *Bacillus* genus have been studied extensively as potential biocontrol agents (BCAs). Bacteria that belong to the *Bacillus* genus have a high replication rate, endospores which enhance their ability to survive under stressful conditions and have a broad spectrum activity; hence they are the most studied BCAs (Samara *et al*., 2021). Zouari *et al*., (2016) showed that *B. amyloliquefaciens* could protect tomato (*Solanum lycopersicum* L.*)* plants against damping-off caused by *P. aphanidermatum*. In a study conducted by Zhang *et al*., (2020), a non-pathogenic strain of *Ralstonia solanacearum* significantly reduced bacterial wilt disease incidence and severity in tomato plants under greenhouse conditions.

Some of these BCAs are endophytes, which are microorganisms that can be found within plant tissues. They occupy belowground and aboveground plant tissues forming the plant microbial endo-sphere (Fadiji and Babalola, 2020). Endophytes interact with

their host plants in different ways and may be another option in controlling plant diseases other than the general application of fungicides (Orozco-Mosqueda and Sontoyo, 2021). For example, the bacterial endophyte *Pseudomonas putida* isolated from the roots of black pepper (*Piper nigerum* L.) showed antagonistic effects against *Phytophthora capsici, P. myoritylum* and *R. solani* (Agisha *et al*., 2017). Therefore, endophytes can be developed and used in plant disease management (Fadiji and Babalola, 2020).

This study aimed to evaluate selected bacterial endophytes isolated from a medicinal plant (*Arctotis arctotoides* (L.f) O. Hoffm) against *Pythium* spp. and *R. solani* root rot pathogens of maize under greenhouse conditions.

3.2 Materials and Methods

3.2.1 Preparation of pathogen inoculum

About 200 barley seeds were first soaked overnight in 150ml of distilled water. The distilled water was then drained off the seeds, and the seeds were sterilized in the autoclave at 121°C for 15 minutes once a day for two consecutive days. After cooling, the barley seeds were then inoculated with seven-day old agar plugs of *R. solani* and *Pythium* spp. (Kidane, 2008). The inoculated barley seeds were incubated at 25°C for two weeks, carefully dispensed onto a clean tray, and air-dried on a laminar flow bench. Once air-dried, the colonised barley seeds were packed into paper bags and later kept at ambient temperature in the laboratory until needed.

3.2.2 Bacterial suspension

For each pathogen, the best ten bacterial endophytes that had inhibitory effects in the dual culture assays (Chapter 2) were evaluated in the greenhouse. Bacterial endophytes were grown in nutrient agar (NA) plates from stock cultures and incubated for 24 hours at 25°C. Bacterial suspension was prepared by transferring single colonies from the agar plate into 10ml of double sterilised distilled water in 30ml McCartney bottles. The concentration of the bacterial suspensions were adjusted to 1 X 10^6 cfu/ml using serial dilution and plate count methods. The bacterial suspensions were prepared 30 minutes before seed treatments.

3.2.3 Seed treatments

Untreated maize seeds cultivar (DKC 93-74RGEr1) obtained from Monsanto (Pty) Ltd Sandton, Republic of South Africa were surface sterilised with 70% ethanol for one

minute, then rinsed two times with sterile distilled water. The seeds were placed in petri dishes and allowed to air-dry on the laminar flow bench for one hour. Five seeds were then immersed in 10ml of each bacterial suspension in 30ml McCartney bottles for 30 minutes before planting. For treatment against *R.* solani, the bacterial suspensions were; *Stenotrophomonas maltophilia* (NYL15), *Bacillus* spp. (NYR2), *Proteus vulgaris* (NYR13), *Morganella morganii* DG56-16 (NYS3), *Proteus mirabilis* (NYR9)*, Morganella morganii* KC-Tt-01 (NYL20), *Pseudomonas putida* (NYR14), *Bacillus cereus* (NYR11), *Morganella morganii* L143 (NYR3), *Lysinibacillus pakistanensis* (NYL21). For treatment against *Pythium* spp., the bacterial suspensions were; *Alcaligenes faecalis* (NYS7)*, M. morganii* OG003 (NYL14), *M. morganii* L143 (NYR3), *M. morganii* AR_0133 (NYL12), *Myroides odoratus* 6G (NYL18), *Serratia marcescens* (NYS8), *Ralstonia* spp. (NYR8), *P. putida* (NYL16), *B. cereus* (NYR11) and *Bacillus* spp. (NYS9). For both the pathogen inoculated (C + P) and uninoculated controls (C), five seeds were immersed in 10ml sterile distilled water in 30ml McCartney bottles for 30 minutes before planting.

3.2.4 Planting

The experiments were conducted using 5L planting pots filled with filtered sand. Prior to the planting of treated maize seeds, four barley seeds colonised with the pathogen were placed into each pot approximately 4cm below the surface and at equal-distances from each other. One treated maize seed from each bacterial endophyte was separately planted in the middle of the pathogen inoculated pots approximately 4cm below the surface. The pathogen inoculum was placed 3cm away from the maize seed. There were two controls. The uninoculated control pots were without pathogen inoculations while the pathogen inoculated control pots had the pathogen inoculum.

Each pot was supplemented with 5g NPK fertiliser 2:3:4 (30) plus 0.5% Zn from Omnia Nutriology, Bryanston, South Africa, at planting. In addition to this, 5g of LAN (28) Sasol, Sandton, South Africa (Pty) Ltd was added in each pot every fortnight. The experiments were conducted in the greenhouse with an average maximum temperature of 29°C during the day and an average minimum of 19°C at night. There were three replications for each bacterial treatment and the controls. Each experiment was allowed to run for six weeks and data was collected.

3.2.5 Data collection and analysis

Plant height was measured weekly using the Stanley Power Lock 5m measuring tape. The root length was measured at harvesting using a 50cm ruler. Fresh root and shoot weight were also measured at harvesting using the Radwag analytical balance with maximum capacity of 520g. The number of root lesions was visually counted at harvesting. The experiments for *Pythium* spp. and *R. solani* were repeated once. Analysis of variance was done on all parameters and means were separated using Duncan's Multiple Range Test at 5% significance level in GenStat 18th edition.

3.3 Results

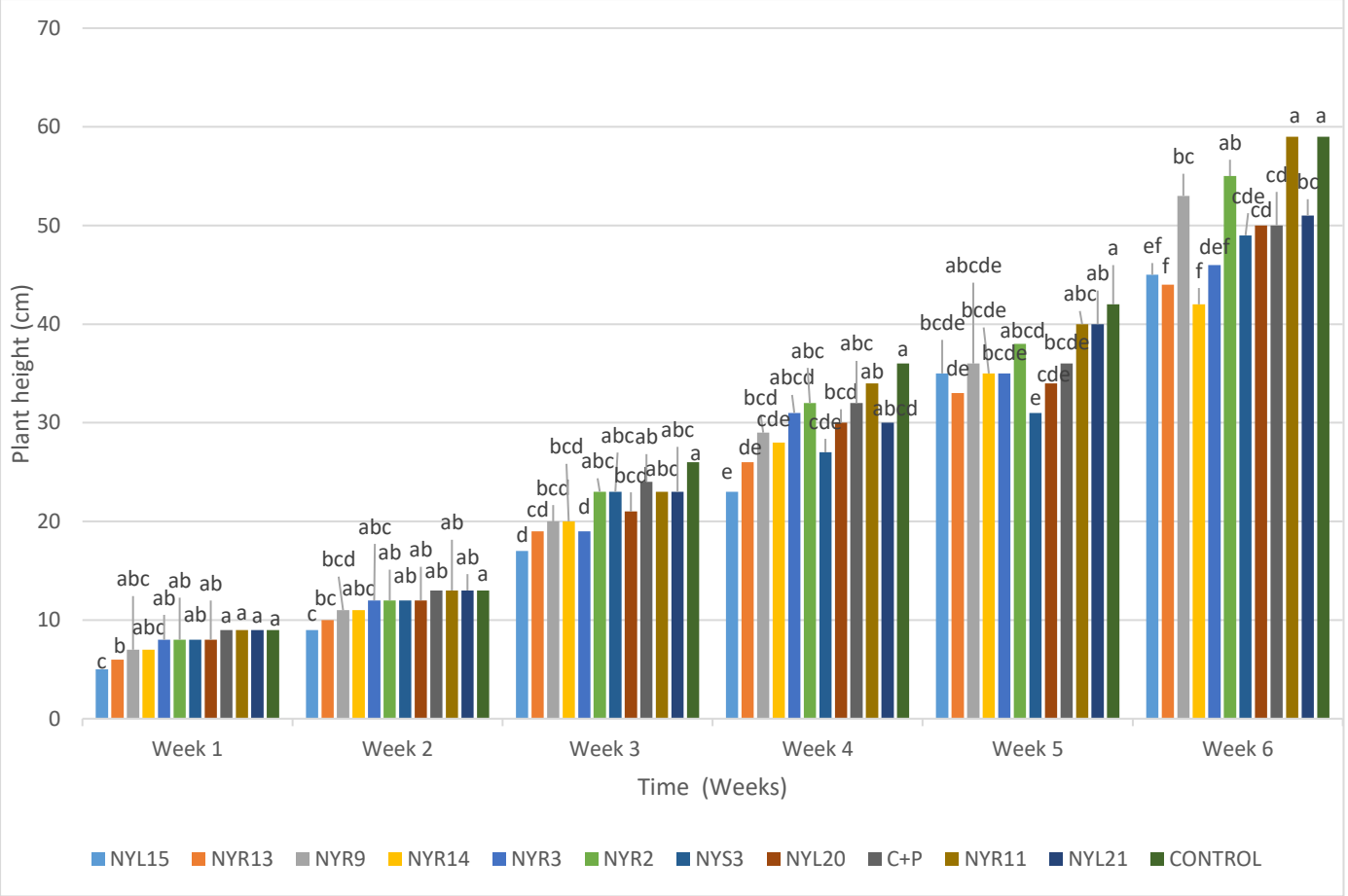
3.3.1 Effect of endophytes on *R. solani* root rot.

3.3.1.1 Plant height

Maize plant height was measured once every week for six weeks, as one of the parameters in the experiments. The results are illustrated in Figures 3.1 and 3.2.

In Experiment 1 (Figure 3.1), maize plants treated with *B. cereus* NYR11 had a significantly higher plant height than other endophytes starting from Week four onwards. At the end of the experiment (Week six), the bacterial endophytes *B. cereus* NYR11, *Bacillus* spp. NYR2 and *P. mirabilis* NYR9 were the best three endophytes with mean plant height of 58.67cm, 55.00cm and 52.67cm respectively.

The performance of bacterial endophytes was not consistent in both experiments. In Experiment 2 (Figure 3.2), the bacterial endophyte *S. maltophilia* NYL15 was consistently the best endophyte treatment every week. In this experiment the best three bacterial endophyte treatments were *S. maltophilia* NYL15, *P. putida* NYR14 and *M. morganii* KC-Tt-01 NYL20 with 108.67cm, 99.67cm and 99.67cm respectively in Week 6. *S. maltophilia* NYL15, *P. putida* NYR14 and *M. morganii* KC-Tt-01 NYL20 differed by 20.67cm, 11.67cm and 11.67cm, respectively, from the pathogen inoculated control. Even though in this experiment, *B. cereus* NYR11 was not among the best three endophytes at harvest, the plant height of maize plants treated with this bacterial endophyte was better than plants treated with *P. putida* NYR14 in weeks four and five.



*There is no significant differences between bars with the same letters according to Duncan's Multiple Range Test at 5% significance level.

Figure 3.1: Weekly plant heights of bacterial endophyte treated maize plants inoculated with *R. solani* measured for six weeks in Experiment 1.

KEY:

NYL15 – *Stenotrophomonas maltophilia*

NYR13 – *Proteus vulgaris*

NYR9 – *Proteus mirabilis*

NYR14 – *Pseudomonas putida*

NYR3 – *Morganella morganii* L143

Control – Uninoculated control

NYR2 – *Bacillus* spp.

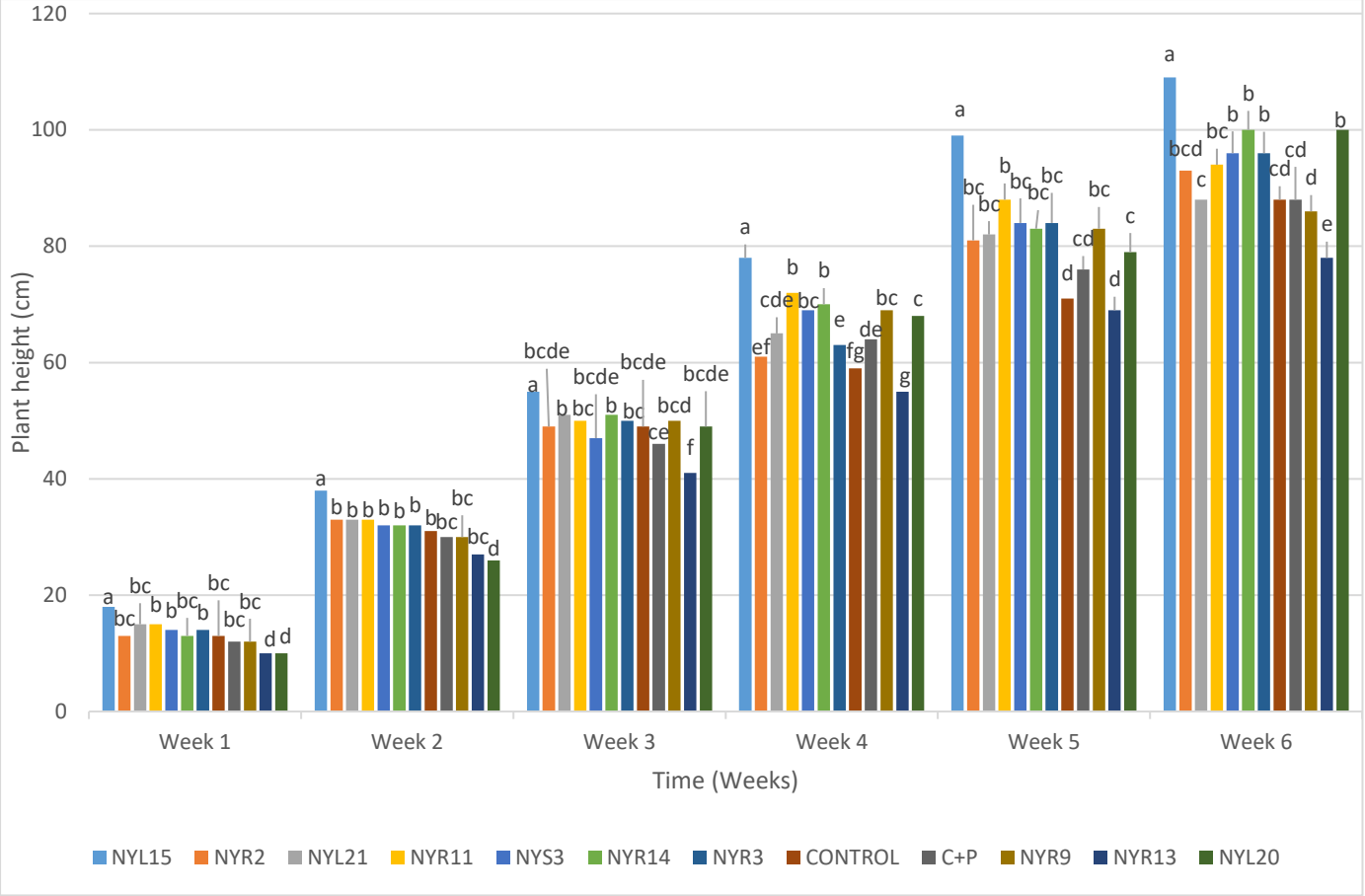
NYS3 – *Morganella morganii* DG56-16

NYL20 – *Morganella morganii* KC-Tt-01

NYR11 – *Bacillus cereus*

NYL21 – *Lysinibacillus pakistanensis*

C + P – Inoculated control



*There is no significant differences between bars with the same letters according to Duncan's Multiple Range Test at 5% significance level.

Figure 3.2: Weekly plant heights of bacteria endophyte treated maize inoculated with *R. solani* measured for six weeks for Experiment 2.

KEY:

NYL15 – *S. maltophilia*

NYR2 – *Bacillus* spp.

NYL21 – *L. pakistanensis*

NYR11 – *B. cereus*

NYS3 – *M. morganii* DG56-16

Control – Uninoculated control

NYR14 – *P. putida*

NYR3 – *M. morganii* L143

NYR9 – *P. mirabilis*

NYR13 – *P. vulgaris*

NYL20 – *M. morganii* KC-Tt-01

C + P – Inoculated

3.3.1.2 Root length

There was a significant difference (P<0.05) between the treatments regarding the root length as shown in Table 3.1. In Experiment 1, the mean root length of maize plants treated with *S. maltophilia* NYL15 was longer than other bacterial endophyte treatments. The mean root length of this bacterial endophyte was also significantly different from the pathogen inoculated control. However, in Experiment 2, treatment of maize plants with *M. morganii* DG56-16 NYS3 showed the highest mean root length (50.00cm) and was significantly different from the rest of the treatments and from the pathogen inoculated control (39.67cm).

Table 3.1 Mean root length (cm) of maize plants from Experiment 1 and 2.

Treatment	**Mean root length (Exp. 1)**	**Mean root length (Exp. 2)**
M. morganii DG56-16 NYS3	23.00c	50.00a
C + P (Inoculated control)	22.67c	38.00b
B. cereus NYR11	23.67c	36.67b
P.vulgaris NYR13	24.00c	28.00c
L. pakistanensis NYL21	24.67c	37.67b
P. putida NYR14	24.67c	35.00b
M. morganii L143 NYR3	24.67c	37.00b
M. morganii KC-Tt-01 NYL20	26.00c	41.00b
Bacillus spp. NYR2	26.00c	36.67b
P. mirabilis NYR9	27.00b	40.00b
S. maltophilia NYL15	30.00ab	36.67b
Control (Uninoculated control)	37.67a	39.67b
P Value	<.001	<.001
L.S.D	3.62	5.3
S.E.D	1.7	2.6
CV %	8.1	8.3

*Mean root length of 3 replicates per treatment. Mean root length with the same letters in each experiment are not significantly different from each other according to Duncan's Multiple Range Test (DMRT) at 5% significance level.

Figure 3.3 shows that the endophyte *B. cereus* NYR11 has the ability to inhibit *R. solani* as the root weight and length were better than that of the pathogen inoculated control.

Figure 3.3: Root system of plants from greenhouse experiment. Differences in root length and weight between maize plants treated with bacterial endophyte *B. cereus* NYR11, uninoculated control and pathogen inoculated control.

3.3.1.3 Root lesions

In both experiments, the endophytes significantly ($P<0.05$) reduced the number of root lesions on maize plants' roots. Maize plants inoculated with *R. solani* showed dark-brown root lesions. In Experiment 1 (Table 3.2), treatment with *P. vulgaris* NYR13, *P. mirabilis* NYR9 and *M. morganii* DG56-16 NYS3 showed the least number of root lesions compared to the pathogen inoculated control. Each of these bacterial endophytes reduced the number of root lesions by 56.28% when compared to the pathogen inoculated control.

In Experiment 2, maize plants treated with *P. mirabilis* NYR9, *B. cereus* NYR11 and *P. vulgaris* NYR13 significantly reduced the number of root lesions compared to the pathogen inoculated control. The endophytes *P. mirabilis* NYR9, *B. cereus* NYR11 and *P. vulgaris* NYR13 reduced the number of root lesions by 46.21%, 40.21% and 40.21%, respectively.

Table 3.2: Mean number of root lesions in maize plants inoculated with *R. solani*

Treatment	**Mean no. of lesions (Exp. 1)**	**Mean no. of lesions (Exp. 2)**
P. vulgaris NYR13	2.33b	1.41cd
P. mirabilis NYR9	2.33b	1.28d
*M. morganii DG56-16*NYS3	2.33b	2.00ab
M. morganii KC-Tt-01 NYL20	2.67b	1.98ab
L. pakistanensis NYL21	2.67b	1.62bcd
B. cereus NYR11	2.67b	1.41cd
Bacillus spp. *NYR2*	2.67b	1.79bc
S. maltophilia NYL15	3.00b	1.47cd
P. putida NYR14	3.00b	1.73bc
M. morganii NYR3	3.00b	1.62bcd
C + P (Inoculated control)	5.33a	2.38a
Control (Uninoculated control)	0.00c	0.00e
P value	<.001	<.001
L.S.D	0.74	0.39
S.E.D	0.35	0.19
CV %	16.3	15.1

*Mean number of root lesions of 3 replicates per treatment. Mean number of root lesions with the same letters in each experiment are not significantly different from each other according to Duncan's Multiple Range Test (DMRT) at 5% significance level.

3.3.1.4 Root and shoot weight

Table 3.3 illustrates the mean root and shoot weight of maize plants treated with the different bacterial endophytes. There were significant differences ($P<0.05$) between the treatments in both experiments. For varieties of- root and shoot weight, the performance of the bacterial endophytes was not consistent in Experiment 1 and 2.

In Experiment 1 (Table 3.3), the mean root weights of maize plants treated with *M. morganii* DG56-16 NYS3 and *B. cereus* NYR11 were significantly higher than pathogen inoculated control and the uninoculated control. However, this was not consistent in Experiment 2. In Experiment 2, the root weight of maize plants treated with *M. morganii* L143 NYR3, *P. mirabilis* NYR9, *P. putida* NYR14, *B. cereus* NYR11 and *M. morganii* DG56-16 NYS3 was significantly ($P<0.05$) higher than the pathogen inoculated control and the uninoculated control.

The mean shoot weight of maize plants treated with *B. cereus* NYR11 in Experiment 1 was significantly different from other treatments, including all controls and was the highest mean root weight. In Experiment 2, treatment with *S. maltophilia* NYL15 was the best and was significantly different from the other treatments. Both treatments with *B. cereus* NYR11 and *S. maltophilia* NYL15 in Experiment 1 and 2 respectively, were significantly different from the pathogen inoculated control.

Table 3.3 Mean root and shoot weight (g) of maize plants measured over six weeks after treatments with endophytic bacterial strains in the presence of *R. solani*.

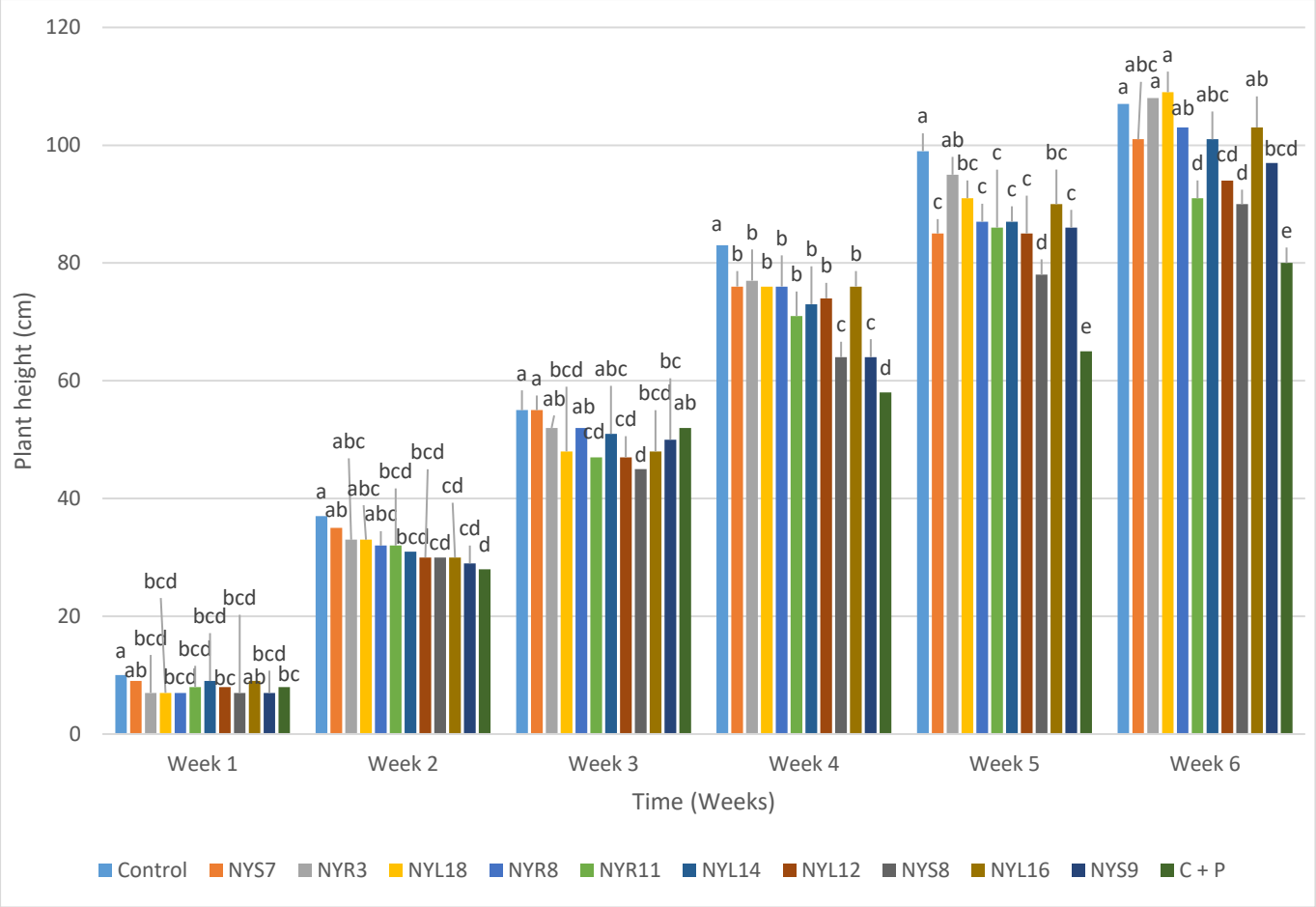
Treatment	**Root weight (g)**	**Root weight (g)**	**Shoot weight (g)**	**Shoot weight (g)**
	Exp. 1	**Exp. 2**	**Exp. 1**	**Exp. 2**
M. morganii L143 NYR3	0.37e	12.00a	5.32b	50.00bcd
P. mirabilis NYR9	0.41e	10.67a	5.27b	59.67ab
P. putida NYR14	0.44de	11.00a	2.67e	48.67cd
Bacillus spp. NYR2	0.44de	5.67c	3.51cde	41.67d
S. maltophilia NYL15	0.47cde	8.33b	3.57cde	63.00a
P. vulgaris NYR13	0.47cde	5.00c	4.41bcd	18.00e
C+ P (Inoculated control)	0.48cde	4.67c	3.61cde	25.67e
L. pakistanensis NYL21	0.64bc	6.67bc	3.00de	40.67d
M. morganii KC-Tt-16 NYL20	0.68b	7.00bc	5.32b	42.67cd
B. cereus NYR11	0.74b	10.67a	8.55a	49.00cd
M. morganii DG56-16 NYS3	0.93a	11.00a	4.45bc	52.67bc
Control (Uninoculated control)	0.62bcd	7.00bc	5.41b	43.67cd
P value	<.001	<.001	<.001	<.001
L.S.D	0.17	2.2	1.27	9.3
S.E.D	0.08	1.0	0.6	4.5
CV%	17.5	15.5	16.4	12.4

*****Mean triplicate root and shoot weight; means with same letters in the same column are not significantly different from each other at a 5% significant level according to Duncan's Multiple Range Test.

3.3.2 Effect of endophytes on *Pythium* spp. root rot of maize.

3.3.2.1 Plant height

Figures 3.4 and 3.5 illustrate plant height of maize treated with bacterial endophytes against *Pythium* spp. The best three treatments changed every week throughout the experiments. In Experiment 1 (Figure 3.4), *M. morganii* L143 NYR3 was one of treatments which consistently increased plant height in all the six weeks. Moreover, it was among the best three treatments from Week one to Week four in Experiment 2 (Figure 3.5). Even though plants treated with *Myroides odoratus* 6G NYL18 were not consistently in the best three treatments in both experiments, they were in the best three in Weeks five and six in both experiments. By Week six, maize plants treated with *M. odoratus* 6G NYL18 had the best plant height (108.67cm and 106.00cm) compared to other treatments in Experiment 1 and 2 respectively. Compared to the pathogen inoculated control (C + P), treatment of maize plants with the bacterial endophytes increased plant height in both experiments.



*There is no significant differences between bars with the same letters according to Duncan's Multiple Range Test at 5% significance level.

Figure 3.4: Weekly plant heights of bacterial endophyte treated maize plants inoculated with *Pythium* spp. measured over a period of six weeks in Experiment 1.

KEY:

NYS7 – *Alcaligenes faecalis*

NYR3 – *M. morganii* L143

NYL18 – *Myroides odoratus* 6G

NYR8 – *Ralstonia* spp.

NYR11 – *B. cereus*

Control – Uninoculated control

NYL14 – *M. morganii* OG003

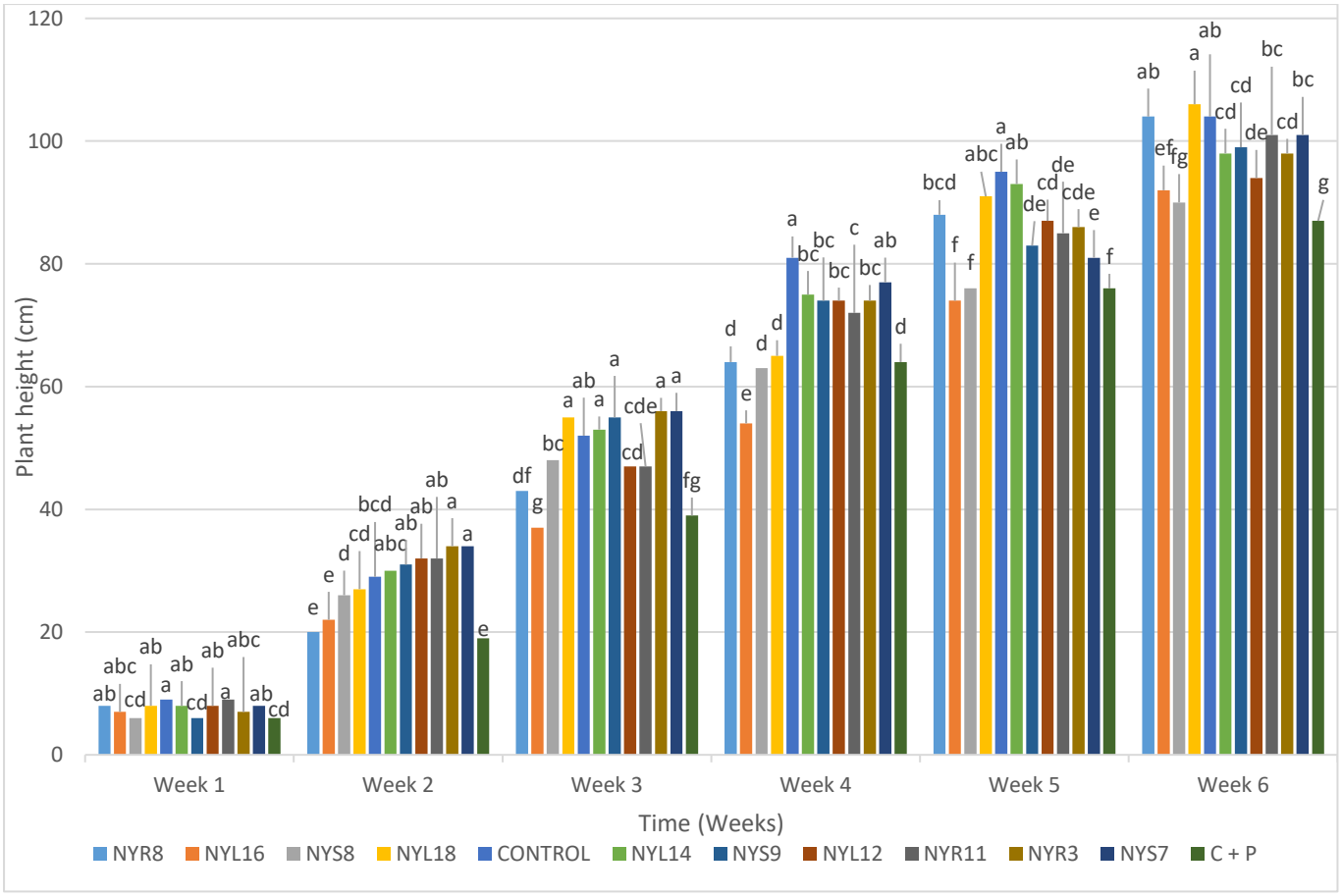
NYL12 – *M. morganii* AR_0133

NYS8 – *Serratia marcescens*

NYL16 – *P. putida*

NYS9 – *Bacillus* spp.

C + P – Inoculated control



*There is no significant differences between bars with the same letters according to Duncan's Multiple Range Test at 5% significance level.

Figure 3.5: Weekly plant heights of bacterial endophyte treated maize plants inoculated with *Pythium* spp. measured over a period of six weeks in Experiment 2.

KEY:

NYR8 – *Ralstonia* spp.

NYL16 – *P. putida*

NYS8 – *S. marcescens*

NYL18 – *M. odoratus*

NYL14 – *M. morganii* OG003

Control – Uninoculated control

NYS9 – *Bacillus* spp.

NYL12 – *M. morganii* AR_0133

NYR11 – *B. cereus*

NYR3 – *M. morganii* L143

NYS7 – *A. faecalis*

C + P – Inoculated control

3.3.2.2 Root length

Root length of maize plants in both experiments was measured. In both experiments, *M. morganii* AR_0133 NYL12 was the treatment with significantly longer roots than most treatments. In Experiment 2, there was no significant difference between *M.*

morganii AR_0133 NYL12, *A. faecalis* NYS7, *Ralstonia* spp. NYR8 as well as the uninoculated control. These bacterial endophytes were also the best three treatments in Experiment 2.

Table 3.4 Mean root length (cm) of maize plants inoculated with *Pythium* spp. after treatment with bacterial endophytes under greenhouse conditions in two separate experiments.

Treatments	**Mean root length (Exp. 1)**	**Mean root length (Exp. 2)**
B. cereus NYR11	28.33g	33.67bc
C + P (Inoculated control)	29.00fg	27.67d
Bacillus spp. NYS9	31.00efg	29.67cd
S. marcescens NYS8	32.67def	34.00b
Ralstonia spp. NYR8	34.00de	42.67a
M. morganii L143 NYR3	34.33de	34.67b
A. faecalis NYS7	34.33de	40.67a
M. odoratus 6G NYL18	35.33cd	36.67b
M. morganii OG003 NYL14	36.00cd	35.33b
P. putida NYL16	41.33b	32.67bc
M. morganii AR_0133 NYL12	49.00a	42.67a
Control (Uninoculated control)	38.67bc	41.00a
P value	<.001	<.001
L.S.D	3.69	3.94
S.E.D	1.78	1.90
CV%	6.2	6.5

*Mean root length of 3 replicates per treatment. Mean root length with the same letters in each experiment are not significantly different from each other according to Duncan's Multiple Range Test (DMRT) at 5% significance level.

Figure 3.6 shows the root system of maize plant treated with bacterial endophyte *Ralstonia* spp. NYR8, healthier than that of maize roots inoculated with *Pythium* spp. without any bacterial endophyte.

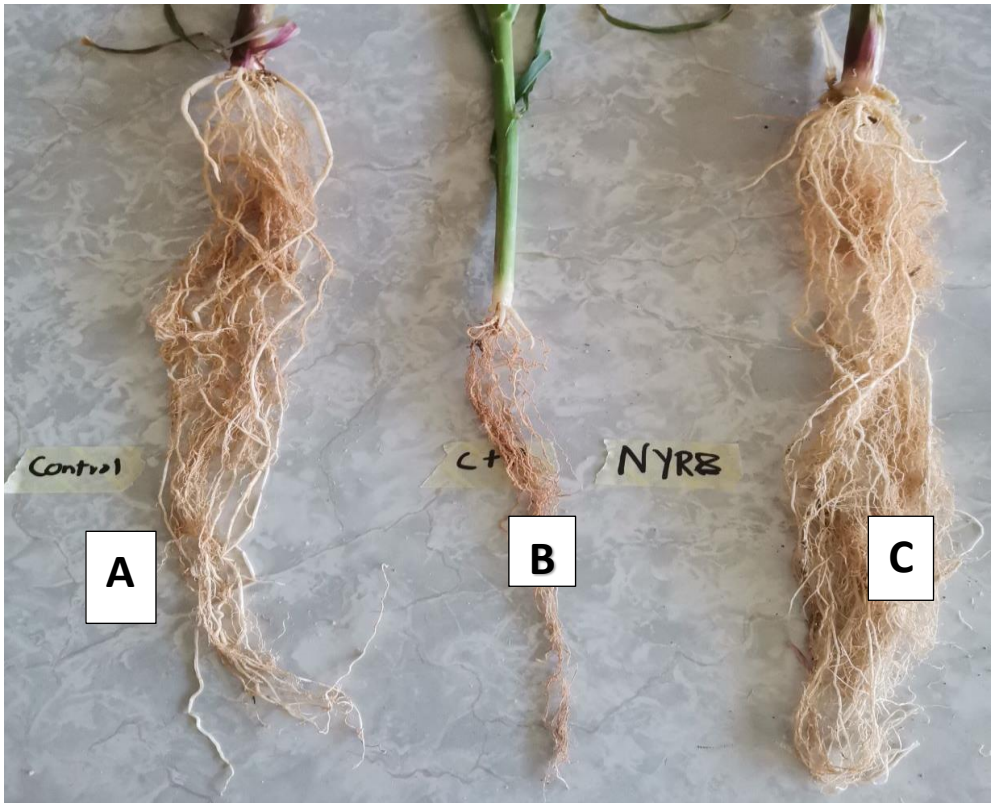


Figure 3.6: (A) Root length of maize in control without pathogen (uninoculated control). (B) control with the pathogen and (C) treatment with bacterial endophyte *Ralstonia* spp. NYR8.

3.3.2.3 Root lesions

Maize plants inoculated with *Pythium* spp. had brown root lesions. The average number of root lesions per treatment is represented in Table 3.5. Treatment of maize plants with the different bacterial endophytes significantly ($P< 0.05$) reduced the number of root lesions in the presence of *Pythium* spp. In both experiments, treatment with *A. faecalis* NYS7 and *Ralstonia* spp. NYR8 resulted in a low number of root lesions. Compared with the pathogen inoculated control, *A. faecalis* NYS7 decreased the number of root lesions by 26.70% and 82.36% in Experiment 1 and 2, respectively. *Ralstonia* spp. NYR8 reduced the number of root lesions by 33.51% and 52.91% in Experiments 1 and 2, respectively.

Table 3.5: Mean number of root lesions in maize plants inoculated with *Pythium* spp. after treatment with bacterial endophytes under greenhouse conditions in two experiments.

Treatments	Mean no. of root lesions (Exp. 1)	Mean no. of root lesions (Exp. 2)
C + P (Inoculated control)	1.91a	5.67a
M. morganii AR_0133NYL12	1.62ab	4.67a
P. putida NYL16	1.62ab	2.67b
M. morganii OG003NYL14	1.52abc	3.33b
M. odoratus NYL18	1.52abc	3.00b
S. marcescens NYS8	1.52abc	5.33a
M. morganii L143 NYR3	1.41bc	3.33b
B. cereus NYR11	1.38bc	5.00a
Bacillus spp. NYS9	1.38bc	4.67a
Ralstonia spp. NYR8	1.27bc	2.67b
A. faecalis NYS7	1.14c	1.00c
Control (Uninoculated control)	0.00d	0.00c
P value	<.001	<.001
L.S.D	0.37	1.04
S.E.D	0.17	0.50
CV%	16.2	17.8

*Mean number of root lesions of 3 replicates per treatment. Mean number of root lesions with the same letters in each experiment are not significantly different from each other according to Duncan's Multiple Range Test (DMRT) at 5% significance level.

3.3.2.4 Root and shoot weight

Root and shoot weight of maize plants were measured in all plants treated with bacterial endophytes in the presence of *Pythium* spp., as well as from the control plants, these are presented in Table 3.6. The average root weight of plants treated with *M. morganii* AR_0133 NYL12, *Ralstonia* spp. NYR8, and *M. odoratus* 6G NYL18 in the second experiment had significantly higher root weight. Seed treatment with all applied bacterial endophytes increased the mean root weight of maize plants compared to the pathogen inoculated control in both experiments. This was also shown by the mean shoot weight. In both experiments, *M. odoratus* 6G NYL18, *A.*

faecalis NYS7 and *Ralstonia* spp. NYR8 were the best three treatments in terms of shoot weight. Seed treatment with these bacterial endophytes remarkably increased the shoot weight of maize plants especially when compared with the pathogen inoculated control.

Table 3.6 Mean root and shoot weight (g) of maize plants treated with different bacterial endophytes.

Treatments	**Mean root weight (g)**	**Mean root weight (g)**	**Mean shoot weight (g)**	**Mean shoot weight (g)**
	Exp. 1	**Exp. 2**	**Exp. 1**	**Exp. 2**
M. morganii AR_0133 NYL12	13.00a	12.00a	53.00de	50.67de
Ralstonia spp. NYR8	11.33a	12.00a	63.00bc	64.00bc
A. faecalis NYS7	8.66b	9.67b	66.67ab	67.33b
M. morganii OG003 NYL14	8.00b	8.67bc	46.67ef	53.00d
P. putida NYL16	8.00b	4.00e	58.67bcd	52.00de
M. odoratus 6G NYL18	8.00b	11.67a	74.67a	77.00a
B. cereus NYR11	8.00b	8.00c	57.00cd	60.67bc
S. marcescens NYS8	7.67b	8.00bc	60.00bcd	45.00e
M. morganii L143 NYR3	7.00b	8.67bc	55.67cd	57.00cd
CONTROL (Uninoculated control)	6.33b	7.33cd	73.00a	75.67a
Bacillus spp. NYS9	3.00c	6.00d	59.00bcd	60.67bc
C + P (Inoculated control)	2.67c	4.00e	41.67f	35.67f
P value	<.001	<.001	<.001	<.001
L.S.D	2.04	1.54	7.82	6.68
S.E.D	0.98	0.74	3.77	3.22
CV%	15.8	10.9	7.8	6.8

*****Mean triplicate root and shoot weight, means with same letters are not significantly different from each other at significant level 5%.

3.4 Discussion

In this study, it was demonstrated that bacterial endophytes isolated from *A. arctotoides* significantly reduced root rot caused by *R. solani* and *Pythium* spp. on maize. Use of bacterial organisms as seed treatments is a common practice in agriculture enhances plant growth and inhibit disease development (Yu and Lee, 2013). It has been reported that endophytes that inhibit the growth of pathogens *in vitro* might show lower abilities to control the pathogen under greenhouse conditions (Wiewiora *et al.,* 2015). This reduced ability in the greenhouse may be caused by the environment, host, pathogen and endophyte interactions under greenhouse conditions (Wiewiora *et al.*, 2015).

In this study, bacterial endophytes were applied as seed treatment which is a common method of application in controlling root rot diseases. There were significant differences ($P<0.05$) between treatments in all experiments in this study. However, the plant height, root length, mean root and shoot weight of plants in Experiment 1 of *R. solani* inoculation were lower than *R. solani* Experiment 2, *Pythium* spp. Experiment 1 and 2. This can be attributed to the fact that application of LAN (28) Sasol, Sandton, South Africa (Pty) Ltd was delayed and applied in week 3 of this experiment as compared to week 2 application for other experiments.

Bacterial endophytes have been reported to promote plant growth through production of lytic enzymes that inhibit pathogenesis of plant pathogens and inciting plant defence mechanisms (Morale-Cedeno *et al*., 2021). Endophytes can also stimulate plant growth by producing indole acetic acid, some endophytes are also involved in the nitrogen fixation process (Morale-Cedeno *et al*., 2021). In this study, various endophytes promoted plant height compared to the pathogen inoculated control. In *R. solani* inoculated plants, *B. cereus* NYR11, *Bacillus* spp. NYR2, and *P. mirabilis* NYR9 in Experiment 1, and *S. maltophilia* NYL15 and *P. putida* NYR14 and *M. morganella* KC-Tt-01 NYL20 in Experiment 2 promoted plant growth. In maize plants inoculated with *Pythium* spp., *M. morganii* L143 NYR3 and *P. putida* NYL16 promoted plant growth in Experiment 1, while *Ralstonia* spp. NYR8 and *A. faecalis* NYS7 increased plant height in Experiment 2. *M. odoratus* 6G NYL18 was in the top three treatments that promoted plant growth in both experiments. Endophytes which belong to the *Bacillus* and *Pseudomonas* genus have been reported to promote plant growth (Morale-Cedeno *et al*., 2021), as seen in the results of this study. A NapA gene

responsible for phosphorous solubilisation was isolated from *M. morganii* and transferred to *Burkholderia cepacia* IS-16 which was used as a biofertilizer to increase phosphate solubilisation, a trait involved plant growth promotion (Rodriguez *et al*., 2006).

In a study conducted by Karadeniz *et al*., (2006), it was found that *P. mirabilis* can produce indole acetic acid and gibberellins which promote plant growth. *P. mirabilis* NYR9 promoted root length in this study as it was one of the top three treatments with higher root length on maize plants inoculated with *R. solani*. The root length of maize plants inoculated with *R. solani* was also promoted by treatment with *M. morganii* KC-Tt-01 NYL20 in both experiments. *M. morganii* AR_0133 NYL12 promoted root length in the presence of *Pythium* spp. It has been reported that *P. vulgaris* JBLS202 promotes the shoot and root length of cabbage (*Brassica oleracea var. capita*) plants by 40.8% and 26.3% respectively, through production of indole acetic acid (Yu & Lee., 2013). However, *P. vulgaris* NYR13 evaluated in this study did not promote plant height and root length when compared to both the pathogen inoculated and uninoculated controls in the presence of *R. solani.*

It has been reported that treatment of tomato plants with *A. faecalis* reduced disease incidence of damping-off caused by *P. aphanidermatum* (Karunasinghe *et al*., 2020). Karunasinghe *et al*., (2020) also reported that *A. faecalis* promoted shoot length and seedling vigour in tomato plants under greenhouse conditions. Seed treatment of maize plants with *A. faecalis* NYS7 in the presence of *Pythium* spp. promoted plant height and root length compared to the pathogen inoculated control in this study. The bacterial endophyte *A. faecalis* NYS7 isolated in this study also reduced the number of root lesions.

A number of studies have reported antagonism of the *Bacillus* genera against plant pathogens (Zouari *et al*., 2016*,* Samaras *et al*., 2021). Bacteria in the *Bacillus* genera have the ability to reduce the disease severity of *R. solani* causing root rot in tomato plants *in vivo* (Abbas *et al*., 2019). The *Bacillus* genera produces numerous secondary metabolites and hydrolytic enzymes involved in their antagonistic activity (Solanki *et al*., 2015). *Bacillus* spp. also produce antimicrobial peptides such as bacillomycin, iturin and surfactin to suppress plant diseases. In this study, maize plants treated with *B. cereus* NYR11 and *Bacillus* spp. NYR2, reduced the number of root lesions

compared to the pathogen inoculated control in *R. solani* experiments. Kumar *et al*., (2021), reported that *B. cereus* can reduce root rot symptoms caused *Macrophomina phaseolina* in *Vigna mungo* L.

Bacterial endophytes in this study significantly reduced the number of root lesions compared to the pathogen inoculated control in the *R. solani* and *Pythium* spp. experiments. Root lesions are typical symptoms of root rot pathogens, and they usually integrate leading to collapse of the whole plant (Jacobs *et al*., 2019). Seed treatment of maize plants with some of the bacterial endophytes in this study also resulted in mean root length higher than the pathogen inoculated control, e.g. *B. cereus* NYR11 in the presence of *R. solani* and *Ralstonia* spp. NYR8 against *Pythium* spp. Bacterial endophytes that were able to suppress root rot symptoms potentially produce secondary metabolites and antimicrobial enzymes that inhibit the growth of the pathogen (Solanki *et al*., 2015).

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CHAPTER 4: Combinations of bacterial endophytes as seed treatments against *Pythium* spp*.* and *Rhizoctonia solani* on maize under greenhouse conditions.

Abstract

Plant disease management strategies such as soil solarisation and cultural practices are usually ineffective. In this study, the best three bacterial endophytes *Bacillus cereus* NYR11, *Morganella morganii* DG56-16 NYS3 and *Proteus mirabilis* NYR9 from previous greenhouse trials were used as seed treatments against *R. solani* on maize. *Alcaligenes faecalis* NYS7, *Ralstonia* spp. NYR8 and *Myroides odoratus* NYL18 were used against *Pythium* spp. The six endophytes and their combinations were used to manage the pathogens. Their efficacy in root rot management was determined by measuring root length, root and shoot weight, plant height, and number of root lesions. Mixtures of *Bacillus cereus* NYR11 with *Morganella morganii* DG56-16 NYS3, and *Proteus mirabilis* NYR9 with *M. morganii* DG56-16 NYS3, significantly reduced the number of root lesions, increased root length and root weight in the presence of *R. solani.* In maize plants inoculated with *Pythium* spp., the single applications of *Ralstonia* spp. NYR8 and *Myroides odoratus* 6G NYL18 were better treatments than their mixtures. These endophytes, especially *M. odoratus* 6G NYL18 increased root length, root and shoot weight, reduced the number of root lesions when applied individually. Therefore, the endophytic bacterial mixtures except for *M. odoratus* 6G NYL18 + *Alcaligenes faecalis* NYS7 + *Ralstonia* spp. NYR8 used as seed treatments against *Pythium* spp. did not control root rot better than the single applications of individual bacteria endophytes. The potential modes of action of the bacterial endophytes were evaluated using agar plates made up of media adjusted for the evaluation of protease, cellulose, chitinase and siderophores production separately. *M. morganii* DG56-16 NYS3, *A. faecalis* NYS7, *P. mirabilis* NYR9, *B. cereus* NYR11 and *M. odoratus* NYL18 produced cellulase and siderophores.

4.1 Introduction

Plant disease management strategies such as soil solarisation and cultural practices have been used for decades. However, these methods are mostly not effective (Abdeljalil *et al*., 2021). Fungicide application is the most common method used to control plant diseases. In most cases agrochemicals are usually expensive and

hazardous. Consistent application of fungicides may also lead to the development of fungicide resistance by the pathogen (Abdeljalil *et al*., 2021). Therefore, biocontrol agents (BCAs) can be used as an alternative. Their modes of action include triggering induced systemic resistance (ISR) and secretion of secondary metabolites such as siderophores, lytic enzymes (protease, chitinase, cellulase, etc.) and antibiotics (Bahmani *et al*., 2021, Li *et al*., 2021).

Medicinal plants are hosts to a distinctive microbiome and are an excellent source for bioactive compounds which can be applied in agriculture, medical and pharmaceutical fields (Zohair *et al*., 2018). Previous studies show that endophytes from medicinal plants are involved in the production of secondary metabolites in their host plants. These endophytes impact the functioning of antioxidant enzymes, resulting in activated defence mechanisms against pathogens (Abdulazeez *et al*., 2020). Even though the functioning of endophytic bacteria is not fully understood, numerous studies have suggested that endophytic bacteria have the ability to promote plant growth and act as BCAs (Bahmani *et al*., 2021).

Fungicides generally have higher efficacy compared to BCAs, and this means more ways should be explored to improve performance of BCAs (Rivas-Garcia *et al*., 2019). In previous studies, it has been shown that a consortium of microorganisms is a better treatment in plant disease management compared to individual applications (Rivas-Garcia *et al*., 2019, Abdeljalil *et al*., 2021). Mutual disease suppression as a result of secondary metabolites produced by one of the organisms towards the pathogen has been reported an effective method used by a consortium of BCAs (Szczech and Shoda., 2004). According to Nunes (2012), a mixture of BCAs controlled *Fusarium proliferatum* better than single BCAs both *in vivo* and *in vitro*. In a study conducted by Szczech and Shoda (2004), it was shown that a mixture of *Bacillus subtilis* RB14-C and *Burkholderia cepacia* BY strains improved *R. solani* control in tomato (*Solanum lycopersicum* L.). *Fusarium oxysporum f.* sp *radicis* of tomato can be controlled through a synergistic effect from a *Pseudomonas* spp. and non-pathogenic *F. oxysporum* consortium (Szczech and Shoda., 2004).

Compatibility of the microorganisms used in mixtures of BCAs is an important trait for plant disease management as incompatible microorganisms might be antagonistic towards each other and not the targeted pathogen (Amirthalingam *et al*., 2020).

Microorganisms generally occur in communities, therefore, in a mixture of BCAs, each microorganism provides a specific role beneficial to the host plant (Abdeljalil *et al*., 2021).

BCAs may inhibit pathogenesis of plant pathogens through parasitism. Endophytes inhibit pathogen growth by parasitism through the production of cell wall degrading enzymes and secondary metabolites such as siderophores (Kohl *et al*., 2019). Lytic enzymes such as chitinases, proteases, cellulases and β-1,3-glucanses, lead to openings in the cell walls of plant pathogens, disrupting the cytoplasm (Kohl *et al*., 2019). Production of these enzymes and secondary metabolites is incited after recognition of the pathogen by BCAs (Kohl *et al*., 2019). Therefore, this study was conducted to evaluate the ability of mixtures of bacterial endophytes to control root-rot pathogens of maize under glasshouse conditions and to evaluate their potential modes of action.

4.2 Materials and methods

4.2.1 Greenhouse trials

4.2.1.1 Pathogen inoculum

Barley seeds colonised with the root rot pathogens, *R. solani* and *Pythium* spp. separately, were used for inoculation. About 200 barley seeds were first soaked in 150ml of distilled water overnight. Distilled water was then drained off the seeds, and the seeds were sterilized in the autoclave at 121°C for 15 minutes once a day for two consecutive days. After cooling off, barley seeds were then inoculated with seven-day-old agar plugs of each pathogen separately (Kidane, 2008). The inoculated barley seeds were incubated at 25°C for two weeks, then carefully dispensed onto a clean tray, and air-dried on a laminar flow bench. Once air-dried, the colonised barley seeds were packed into paper bags and later kept at room temperature until needed.

4.2.1.2 Compatibility test of bacterial endophytes

For *R. solani,* three bacterial endophytes, namely *Proteus mirabilis* NYR9, *Morganella morganii* DG56-16 NYS3 and *Bacillus cereus* NYR11 were tested for compatibility. **For** *Pythium* spp., isolates of *Ralstonia* spp. NYR8, *Myroides odoratus* 6G NYL18 and *Alcaligenes faecalis* NYS7 were tested for their compatibility. The bacterial endophytes were selected based on their ability to reduce the mean number of root

lesions, increase plant height, root length, shoot weight and root weight in the previous experiment. Compatibility tests were conducted as described by James and Mathew, (2017). Two bacterial endophytes were cross-streaked on nutrient agar (NA) plates. One bacterial endophyte was streaked horizontally and the other vertically and incubated for 48 hours at 25°C. The endophytes were considered compatible when there was no clear zone at the point of interaction.

4.2.1.3 Bacterial suspension

For each pathogen, the best three endophytes that reduced root rot symptoms in the glasshouse trial (Chapter 3) were evaluated. Bacterial endophytes were grown on NA plates from stock cultures and incubated for 24 hours at 25°C. A suspension was prepared by transferring single colonies from the agar plate to 10ml of double sterilised distilled water in 30ml McCartney bottles. The concentration of bacterial suspension was adjusted to 1 X 10^6 cfu/ml using the serial dilution method. The suspension was mixed gently to formation of a uniform suspension. Mixture suspensions were prepared by mixing 5ml of each bacterial endophyte suspension to make a 10ml suspension. The total volume of the bacterial suspension of three integrated bacterial endophytes was 15ml. There were four bacterial mixtures and three single suspensions for each pathogen.

4.2.1.4 Seed treatments

Untreated maize seeds of cultivar DKC 93-74RGEr1 obtained from Monsanto (Pty) Ltd, Sandton, Republic of South Africa, were used. The seeds were surface sterilised with 70% ethanol for one minute, then rinsed two times with sterilised distilled water, and allowed to air dry on the laminar flow bench in petri dishes for one hour. Five seeds were then immersed in 10ml of each combined or each single bacterial suspensions for 30 minutes, depending on the treatment structure. For both the uninoculated (neither pathogen nor endophyte) and pathogen inoculated controls, the seeds were immersed in 10ml sterile distilled water for 30 minutes and transferred to planting pots immediately.

4.2.1.5 Planting

The experiments were conducted using 5L planting pots filled with filtered sand. Before planting treated maize seeds, four barley seeds colonised with the pathogen were placed into each pot approximately 4cm below the surface and 2cm from each other.

One treated maize seed from each bacterial endophyte and mixed bacterial endophyte suspensions were each planted in the middle of the pathogen inoculated pot approximately 4cm below the surface. There were two controls. The uninoculated control pots were without pathogen inoculations while the pathogen inoculated control pots had pathogen inoculum.

At planting, each pot was supplemented with 5g NPK fertiliser 2:3:4 (30) plus 0.5% Zn from Omnia Nutriology, Bryanston, South Africa. In addition to this, 5g of LAN (28) Sasol, Sandton, South Africa (Pty) Ltd, was added in each pot every fortnight. The experiments were conducted in the greenhouse with an average maximum temperature of 29°C during the day and an average minimum of 19°C at night. There were three replications for each bacterial endophyte and mixed bacterial endophytes treatment and the controls. The plants were irrigated manually using tap water from planting until the end of the experiment. Each experiment was allowed to run for six weeks, and data was collected.

4.2.1.5 Data collection and analysis

Plant height was measured weekly using the Stanley Power Lock 5m measuring tape. The root length was measured at harvest using a 50cm ruler. Fresh root and shoot weight were also weighed at harvest using the Radwag analytical balance with a maximum capacity of 520g. The experiments for *Pythium* spp. and *R. solani* were repeated once. Analysis of variance (ANOVA) was done using Genstat 18th edition. Mean differences were compared using Fischer's Least Significance Difference (LSD) test. Differences were considered significant when $P \leq 0.05$.

4.2.2 Potential mode of action

4.2.2.1 Protease activity

Production of the protease enzyme by bacterial endophytes was screened on skim milk agar. The agar was prepared using the method described by Duchesne, (2017) with some modifications. Skim milk agar was prepared by pouring 250ml of distilled water into 25g of skim milk powder and stirred until the powder dissolved. 5g of agar was mixed with 250ml distilled water and stirred until dissolved. The two mixtures were then added together, autoclaved at 121°C for 15 minutes, and poured into petri dishes.

A single colony of each bacterial culture was plated on skim milk agar plates and incubated at 25°C for 24 hours. A zone of clearance around the bacterial colony indicated protease activity.

4.2.2.2 Cellulase activity

Cellulose degrading activity of the bacterial endophytes was evaluated using carboxymethyl-cellulose (CMC) media. To prepare the media (gL^{-1}): 1.9 K_2HPO_4, 0.94 KH_2PO_4, 1.6 KCL, 1.43 NaCl, 0.15 NH_4Cl, 0.037 MgSO4•7H2O, 0.017g $CaCl_2$•$2H_20$, 0.1 yeast extract and 10 CMC, pH at 7.2 was used. As a solidifying agent, 20gL^{-1} of agar was added. The media was then sterilised at 121°C for 15 minutes and cooled to room temperature for culturing the bacterial isolate (Handique *et al*., 2017).

Broth culture (5µl) of the bacterial endophytes were separately dropped onto petri dishes containing CMC agar and then incubated for 48h at 28°C. The agar plates were then stained with Gram's iodine solution to visualise cellulase activity. This solution stains the agar containing CMC brown and leave areas without CMC clear, "halos". The appearance of clear halos around the drops confirms cellulase activity by bacteria. Each plate was stained with 10µl of the Gram's iodine solution and allowed to sit for 5 minutes until the dye settled into the media (Maki *et al*., 2011).

4.2.2.3 Chitinase activity

Production of chitinase by bacterial endophytes was determined using an agar medium as described by Atlas and Parks (1993). The medium is made up of ($g.L^{-1}$ of distilled water): colloidal chitin, 15.0 (wet weight); yeast extract, 0.5; $(NH_4)_2SO_4$, 1.0; $MgSO_4$•$7H_2O$, 0.25; KH_2PO_4, 1.0; glucose, 0.5 and agar, 20.0, adjusted to pH 8.0. The medium was autoclaved at 121°C for 15 minutes. After 96 hours of incubation, bacterial endophytes with hydrolysis zones were regarded as chitinase positive endophytes.

Colloidal chitin preparation

40g of chitin powder was slowly added with 600ml of concentrated HCl and kept for 60 minutes with vigorous stirring. Chitin was precipitated as a colloidal suspension by adding it slowly to 2L of water at 4-10°C. The suspension was collected by filtration with suction on a coarse filter paper and washed by suspending it in about 5L of distilled water. Washing was repeated 3 times, until the pH of the suspension was 3.5.

4.2.2.4 Siderophores production

The ability of bacterial endophytes to produce siderophores was evaluated on CAS agar medium, the medium was prepared as described (Yobo, 2005; Srimanthi and Ann Suji, 2018). To prepare the CAS agar medium, 5.3g of NaOH, 30.24g of piperazine-N, -N '-bis (2- ethanesulfonic acid) (Merck) and 20g of agar was dissolved in 750ml of distilled water. A stock solution containing ($g.L^{-1}$ distilled of water): KH_2PO_4, 3.0; NaCl, 5.0; NH_4Cl, 10.0 was prepared and 100ml of this solution was added to the first solution. This solution was then sterilised by autoclaving for 15min at 121°C.

The CAS agar medium was allowed to cool down to ±50°C and then filter sterilised solutions of the following were added: casamino acids (10%) (w/v), 30ml; glucose (20%) (w/v), 10ml; thiamine (200 $\mu g.ml^{-1}$), 10 ml; nicotinic acid (200 $\mu g.ml^{-1}$), 10 ml; $MgCl_2$ (1M) (2.03g in 10ml), 1ml and $CaCl_2$ (1M) (5.55g in 500ml), 1ml. The CAS-iron-hexadecyltrimethylammonium (HDTMA) bromide solution was prepared by dissolving 0.06g of CAS in 50ml distilled water and 0.0027g of $FeCl_3 \bullet 6H_2O$ in 10ml of 10mM HCl. These two solutions were added together, and the resulting solution was slowly added to a solution made up of 0.073g of HDTMA bromide dissolved in 40ml distilled water. This solution was then sterilised by autoclaving at 121°C for 15 minutes. After cooling, the CAS-iron-HDTMA bromide solution was added to the agar medium, mixed gently, and poured into 90mm sterile Petri dishes.

Inoculated plates with bacterial endophytes were incubated for six days. Siderophore production was detected as a yellow-orange halo in an otherwise blue medium around the colonies.

4.3 Results

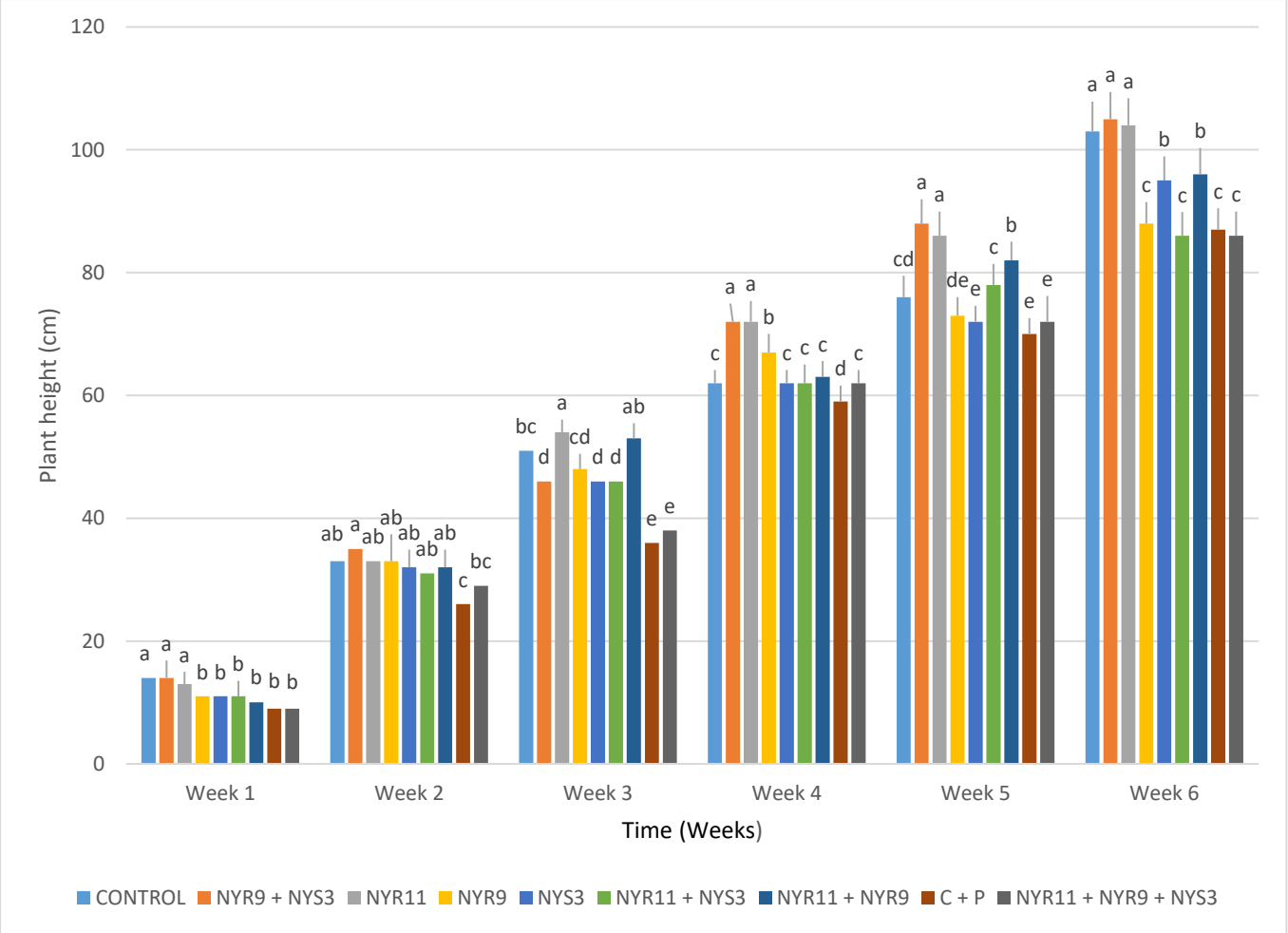
4.3.1 Evaluation of isolates against *R. solani* in greenhouse conditions

4.3.1.1 Plant height

Figures 4.1 and 4.2 below illustrate the weekly plant height of maize plants treated with bacterial isolates in the presence of *R. solani* in Experiments 1 and 2. In Figure 4.1 (Experiment 1), treatment of maize plants with the mixture of *Proteus mirabilis* NYR9 and *Morganella morganii* DG56-16 NYS3 resulted in higher plant height when compared with other isolate combinations every week except Week 3, when *Bacillus cereus* NYR11 mixed with *P. mirabilis* NYR9 had the highest plant height compared

to other combinations. There was no significant difference between the uninoculated control, single application of *B. cereus* NYR11 and *P. mirabilis* NYR9 with *M. morganii* DG56-16 NYS3 during Week 6.

At the end of Experiment 2 (Figure 4.2), mixing the bacterial isolates *B. cereus* NYR11 with *M. morganii* DG56-16 NYS3 resulted in the highest plant height compared with other combinations. The best bacterial combination in Experiment 1 (*P. mirabilis* NYR9 and *M. morganii* DG56-16 NYS3) was the second-best treatment in Experiment 2 following *B. cereus* NYR11 and *M. morganii* DG56-16 NYS3. The best two mixtures were not consistent throughout the experiment, however, *B. cereus* NYR11 mixed with *M. morganii* DG56-16 NYS3 had highest plant height in weeks 2, 3 and 6. In both experiments, combining all three bacterial isolates, *B. cereus* NYR11 + *P. mirabilis* NYR9 + *M. morganii* DG56-16 NYS resulted in low plant height compared with other combinations.



*There is no significant differences between bars with the same letters according to Duncan's Multiple Range Test at 5% significance level.

Figure 4.1 Weekly plant height (cm) of maize plants inoculated with *R. solani* under greenhouse conditions.

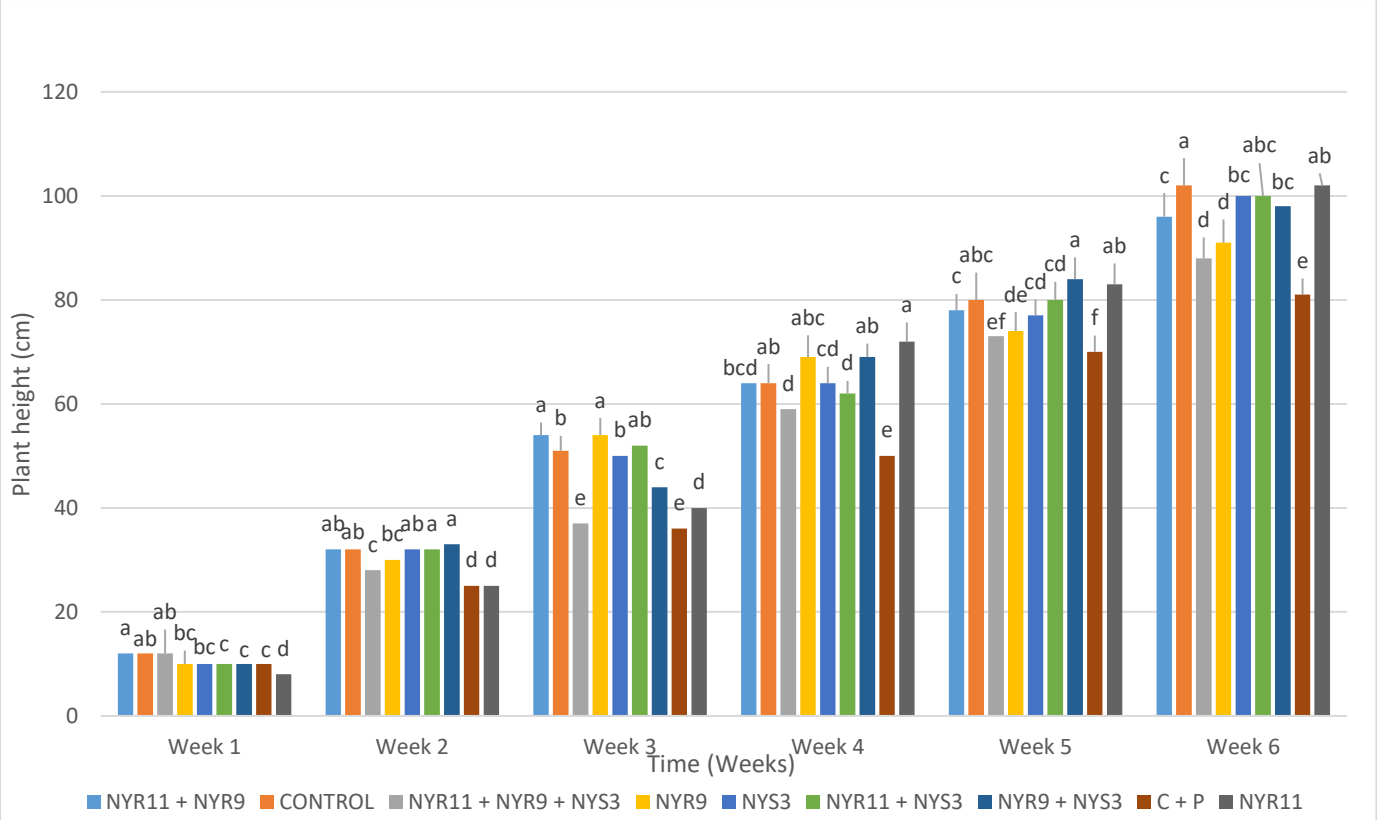
KEY: NYR11 – *Bacillus cereus*

NYR9 – *Proteus mirabilis*

NYS3 – *Morganella morganii* DG56-16

Control – Uninoculated control

C + P – Inoculated control



*There is no significant differences between bars with the same letters according to Duncan's Multiple Range Test at 5% significance level.

Figure 4.2 Weekly plant height (cm) of maize plants inoculated with *R. solani* under greenhouse conditions in Experiment 2.

KEY: NYR11 – *Bacillus cereus*

NYR9 – *Proteus mirabilis*

NYS3 – *Morganella morganii* DG56-16

Control – Uninoculated control

C + P – Inoculated control

4.3.1.2 Root length

Table 4.1 represents the mean root length of maize plants treated with bacterial endophytes in the presence of *R. solani*. There were significant differences in root length between the treatments ($P<0.05$). The performance of the bacterial isolates was not consistent in both experiments, as the best treatment with longest mean root length in Experiment 1 did not have the longest mean root length in Experiment 2.

In Experiment 1, combining *P. mirabilis* NYR9 with *M. morganii* DG56-16 NYS3 significantly increased the mean root length when compared to other combinations. There was no significant difference between *P. mirabilis* NYR9 + *B. cereus* NYR11 + *M. morganii* DG56-16 NYS3 and *B. cereus* NYR11 + *P. mirabilis* NYR9.

In Experiment 2, seed treatment with *B. cereus* NYR11 + *P. mirabilis* NYR9 resulted in longer root length compared to other combinations. Even compared to the single application of the three bacterial isolates, *B. cereus* NYR11 + *P. mirabilis* NYR9 significantly increased root length of maize plants. There was no significance difference between *P. mirabilis* NYR9 + *B. cereus* NYR11 + *M. morganii* DG56-16 NYS3 and the pathogen inoculated control (C + P). In both experiments, the combination of all three bacterial isolates (*P. mirabilis* NYR9 + *B. cereus* NYR11 + *M. morganii* DG56-16 NYS3) resulted in shorter root length when compared with other combinations.

Table 4.1 Mean root length (cm) of maize plants inoculated with *R. solani* under greenhouse conditions.

Treatment	**Exp. 1**	**Exp. 2**
CONTROL (Uninoculated control)	37.00bcd	53.33a
Proteus mirabilis NYR9	35.67cd	39.67de
Bacillus cereus NYR11	36.67bcd	39.00e
Morganella morganii DG56-16 NYS3	40.67bc	41.67cd
B. cereus NYR11 + *M. morganii* DG56-16 NYS3	42.00b	49.33b
B. cereus NYR11 + *P. mirabilis* NYR9	32.67d	52.00a
P. mirabilis NYR9 + *M. morganii* DG56-16 NYS3	53.00a	42.67c
P. mirabilis NYR9 + *B. cereus* NYR11 + *M. morganii* DG56-16 NYS3	32.33d	31.67f
C + P (Pathogen inoculated control)	39.33bc	30.67f
P value	<.001	<.001
L.S.D	5.05	2.54
S.E.D	2.39	2.54
CV%	7.5	3.5

*Mean root length (cm) of three replicates per treatment. Values with the same letters have no significant difference in the same column according to Fischer's LSD at 5% significance level.

4.3.1.3 Root lesions

Table 4.2 below shows the mean number of root lesions in maize plants treated with the bacterial isolate mixtures and single applications in the presence of *R. solani*. There were significant differences (P<0.05), in the mean number of root lesions between the treatments.

In both experiments, seed treatment with *B. cereus* NYR11 + *M. morganii* DG56-16 NYS3 and *P. mirabilis* NYR9 + *M. morganii* DG56-16 NYS3 had the lowest mean number of root lesions. Compared to the combination of all three bacterial isolates and the pathogen inoculated control (C + P), these two treatments significantly reduced the mean number of root lesions.

Table 4.2 Mean number of root lesions of maize plants inoculated with *R. solani* under greenhouse conditions.

Treatment	**Exp. 1**	**Exp. 2**
Control (Uninoculated control)	0.00d	0.00f
P. mirabilis NYR9	3.67bc	5.33b
B. cereus NYR11	3.33bc	3.67d
M. morganii DG56-16 NYS3	3.67bc	3.67d
B. cereus NYR11 + *M. morganii* DG56-16 NYS3	2.67c	2.33e
B. cereus NYR11 + *P. mirabilis* NYR9	3.67bc	5.00bc
P. mirabilis NYR9 + *M. morganii* DG56-16 NYS3	2.67c	2.67e
P. mirabilis NYR9 + *B. cereus* NYR11 + *M. morganii* DG56-16 NYS3	4.33ab	4.33cd
C + P (Pathogen inoculated control)	5.33a	7.33a
P value	<.001	<.001
L.S.D	1.13	0.69
S.E.D	0.53	0.33
CV%	20.0	10.6

*Mean number of root lesions of three replicates per treatment. Values with the same letters have no significant difference in the same column according to Fischer's LSD at 5% significance level.

4.3.1.4 Root weight

Compared to the pathogen inoculated control, treatment of maize seeds with the different bacterial endophytes significantly (P<0.05) increased root weight. In both experiments, seed treatment with a combination of all three bacterial isolates (*P. mirabilis* NYR9 + *B. cereus* NYR11 + *M. morganii* DG56-16 NYS3) resulted in the lowest mean root weight.

In Experiment 1, *B. cereus* NYR11 + *P. mirabilis* NYR9 significantly increased the mean root weight compared to seed treatment with other bacterial isolate combinations. However, in Experiment 2, seed treatment with *B. cereus* NYR11 + *M. morganii* DG56-16 NYS3 significantly increased mean root weight compared to other bacterial combinations.

Table 4.3 Mean root weight (g) of maize plants treated with single endophytes and endophyte consortium against *R. solani*.

Treatment	**Exp. 1**	**Exp. 2**
CONTROL (Uninoculated control)	12.33a	10.67abc
P. mirabilis NYR9	10.00b	11.00ab
B. cereus NYR11	9.33b	9.67bcd
M. morganii DG56-16NYS3	10.67b	9.33cd
B. cereus NYR11 + *M. morganii* DG56-16 NYS3	9.33b	12.00a
B. cereus NYR11 + *P. mirabilis* NYR9	12.67a	10.33bc
P. mirabilis NYR9 + *M. morganii* DG56-16 NYS3	10.67b	9.33cd
P. mirabilis NYR9 + *B. cereus* NYR11 + *M. morganii* DG56-16 NYS3	9.00b	8.67d
C + P (Pathogen inoculated control)	5.33c	7.00e
P value	<.001	<.001
L.S.D	1.52	1.44
S.E.D	0.72	0.68
CV%	8.9	8.5

*Mean root weight (g) of three replicates per treatment. Values with the same letters have no significant difference in the same column according to Fischer's LSD at 5% significance level.

4.3.1.5 Shoot weight

In terms of shoot weight, there were significant differences (P<0.05) between treatments. In Experiment 1, the *B. cereus* NYR11 + *M. morganii* DG56-16 NYS3 seed treatment significantly increased mean shoot weight compared to other treatments. In Experiment 2, *P. mirabilis* NYR9 + *M. morganii* DG56-16 NYS3 significantly increased shoot weight, but there was no significant difference between this treatment and the uninoculated control. In both experiments, there was no significant difference between the *P. mirabilis* NYR9 + *B. cereus* NYR11 + *M. morganii* DG56-16 NYS3 combination and the pathogen inoculated control.

Table 4.4 Shoot weight (g) of maize plants treated with bacterial endophytes under greenhouse conditions.

Treatment	**Exp. 1**	**Exp. 2**
CONTROL (Uninoculated control)	62.67b	63.00a
P. mirabilis NYR9	63.00b	53.67b
B. cereus NYR11	45.00d	42.67d
M. morganii DG56-16 NYS3	61.00bc	54.00b
B. cereus NYR11 + *M. morganii* DG56-16NYS3	69.00a	46.00c
B. cereus NYR11 + *P. mirabilis* NYR9	34.00e	54.00b
P. mirabilis NYR9 + *M. morganii* DG56-16NYS3	55.67c	66.00a
P. mirabilis NYR9 + *B. cereus* NYR11 + *M. morganii* DG56-16 NYS3	39.67def	40.00d
C + P (Pathogen inoculated control)	39.67de	39.67d
P value	<.001	<.001
L.S.D	5.47	3.30
S.E.D	2.58	1.56
CV%	6.1	3.7

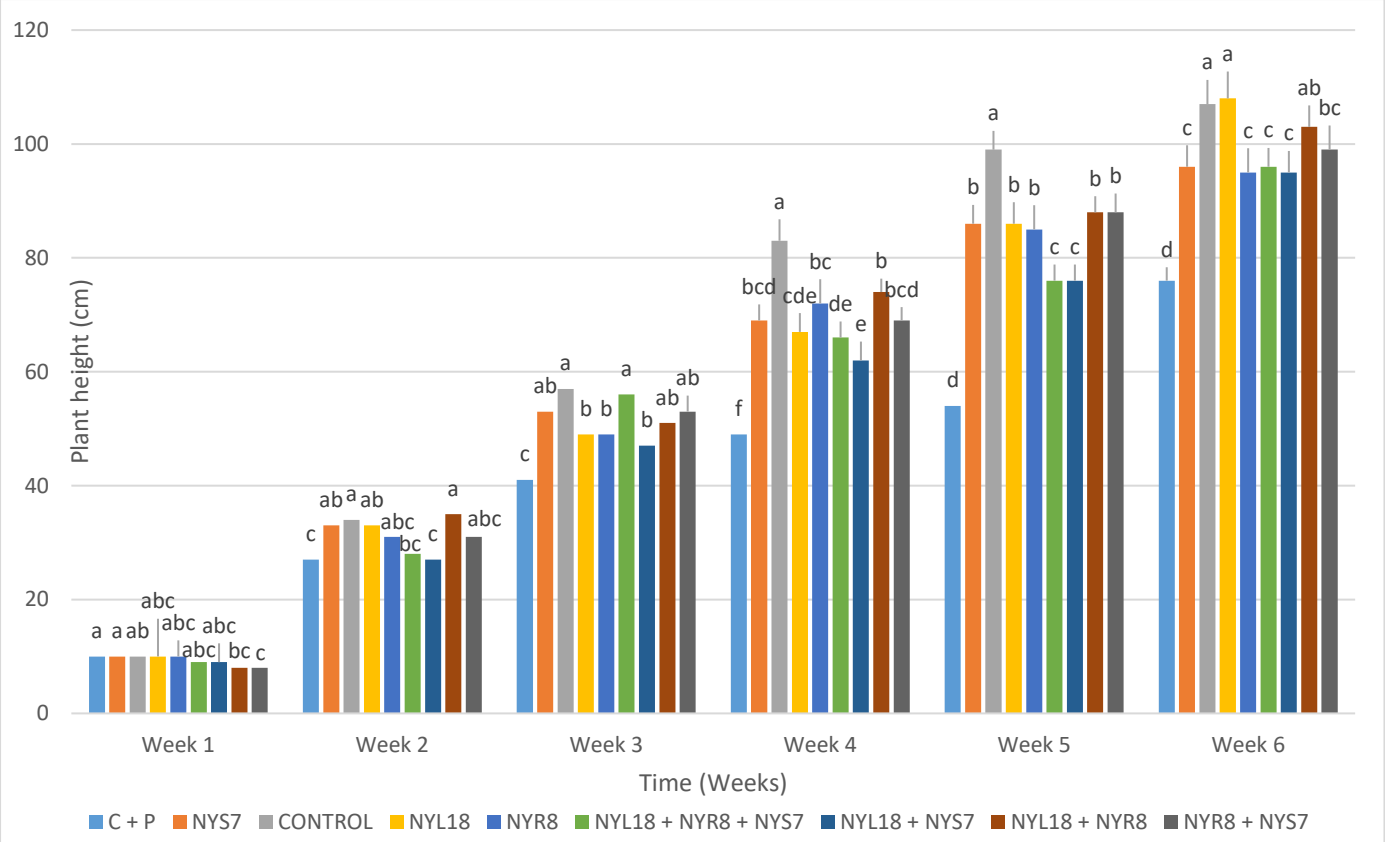
*Mean shoot weight (g) of three replicates per treatment. Values with the same letters have no significant difference in the same column according to Fischer's LSD at 5% significance level.

4.3.2 Evaluation of isolates against *Pythium* spp. under greenhouse conditions

4.3.2.1 Plant height

Figures 4.3 and 4.4 illustrate weekly plant height of maize plants treated with bacterial endophytes and inoculated with *Pythium* spp. Seed treatment with the *M. odoratus* 6G NYL18 + *Ralstonia* spp. NYR8 combination resulted in higher plant height when compared to treatments with other bacterial mixtures. In Experiment 1, maize plants treated with this mixture had a higher plant height than other combinations in Weeks 2, 4, 5, and 6. In Experiment 2, the plant height of maize plants treated with *M. odoratus* 6G NYL18 + *Ralstonia* spp. NYR8 was significantly ($P<0.05$) higher than other bacterial mixtures in Weeks 4, 5, and 6.

During Week 6, there was no significant difference ($P>0.05$) between plant heights of maize plants treated with *M. odoratus* 6G NYL18 + *Ralstonia* spp. NYR8, *Ralstonia* spp. NYL18 and the uninoculated control in both experiments. In both experiments, seed treatment with the bacterial isolates-, significantly increased plant height compared to the pathogen inoculated control (C + P).



*There is no significant differences between bars with the same letters according to Duncan's Multiple Range Test at 5% significance level.

Figure 4.3 Plant height (cm) of maize plants inoculated with *Pythium* spp. in Experiment 1 under greenhouse conditions and treated with different endophytes and their combinations.

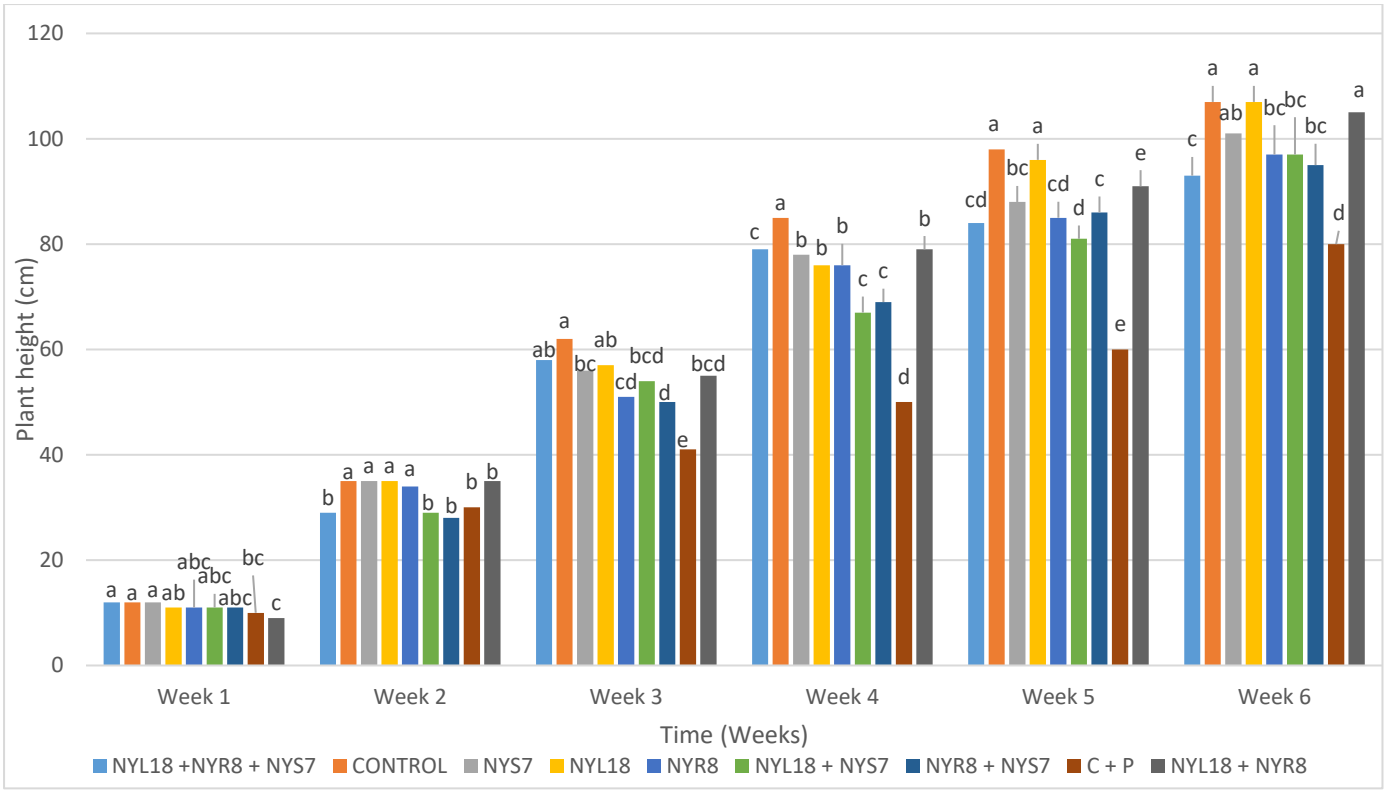
KEY: NYS7 – *Alcaligenes faecalis*

NYL18 – *Myroides odoratus* 6G

NYR8 – *Ralstonia* spp.

C – Uninoculatd control

C + P – Inoculated control



*There is no significant differences between bars with the same letters according to Duncan's Multiple Range Test at 5% significance level.

Figure 4.4 Plant height (cm) of maize plants in Experiment 2 under greenhouse conditions over a period of six weeks, treated with single and combinations of endophytes.

KEY: NYS7 – *Alcaligenes faecalis*

NYL18 – *Myroides odoratus* 6G

NYR8 – *Ralstonia* spp.

C – Uninoculated control

C + P – Inoculated control

4.3.2.2 Root length

Table 4.5 below illustrates the root length of maize plants after a growth period of six weeks. There were significant (P<0.05) differences between the treatments. The root length of maize plants treated with *M. odoratus* 6G NYL18 + *A. faecalis* NYS7 + *Ralstonia* spp. NYR8 was longer than other mixtures in both experiments. The single application of the bacterial endophyte *M. odoratus* 6G NYL18 promoted root length of

maize plants more than treatment with mixed bacterial endophytes-, it was, however, lower than the uninoculated control in both experiments.

Table 4.5 Mean root length (cm) of maize plants for two experiments over a period of six weeks.

Treatment	**Exp. 1**	**Exp. 2**
CONTROL (Uninoculated control)	80.33a	87.00a
Alcaligenes faecalis NYS7	33.00e	35.33de
Ralstonia spp. NYR8	36.00de	36.00d
Myroides odoratus 6G NYL18	56.67b	54.33b
M. odoratus 6G NYL18 + *Ralstonia* spp. NYR8	35.67de	31.33fg
Ralstonia spp. NYR8 + *A. faecalis* NYS7	38.00d	32.67ef
M. odoratus 6G NYL18 + *A. faecalis* NYS7	38.00d	38.00d
M. odoratus 6G NYL18 + *A. faecalis* NYS7 + *Ralstonia* spp. NYR8	42.00c	49.67c
C + P (Pathogen inoculated control)	26.33f	29.33g
P value	<.001	<.001
L.S.D	3.47	3.16
S.E.D	1.64	1.49
CV%	4.7	4.2

*Mean root length (cm) of three replicates per treatment. Values with the same letters have no significant difference in the same column according to Fischer's LSD at 5% significance level.

4.3.2.3 Root lesions

The mean number of root lesions in maize plants inoculated with *Pythium* spp. is represented in Table 4.6 below. There were significant differences ($P<0.05$) between treatments. From the four different bacterial mixtures used in both experiments, the *M. odoratus* 6G NYL18 + *A. faecalis* NYS7 + *Ralstonia* spp. NYR8 mixture had the least number of root lesions. Combination of all three bacterial endophytes significantly ($P<0.05$) reduced the mean number of root lesions compared to the single applications of the three endophytes. The mean number of root lesions in all mixtures was lower than the pathogen inoculated control.

Table 4.6 Mean number of root lesions in maize plants inoculated with *Pythium* spp. under greenhouse conditions.

Treatment	Exp.1	Exp. 2
Control (Uninoculated control)	0.00e	0.00d
A. faecalis NYS7	2.67d	2.33c
Ralstonia spp. NYR8	3.00cd	3.33bc
M. odoratus 6G NYL18	2.67d	3.00bc
M. odoratus 6G NYL18 + *Ralstonia* spp. NYR8	3.67bc	3.67b
Ralstonia spp. NYR8 + *A. faecalis* NYS7	4.33ab	3.67b
M. odoratus 6G NYL18 + *A. faecalis* NYS7	4.00ab	3.67b
M. odoratus 6G NYL18 + *A. faecalis* NYS7 + *Ralstonia* spp. NYR8	2.67d	2.33c
C + P (Pathogen inoculated control)	4.67a	5.33a
P value	<.001	<.001
L.S.D	0.85	1.09
S.E.D	0.40	0.52
CV%	16.1	20.8

*Mean root lesions (cm) of three replicates per treatment. Values with the same letters have no significant difference in the same column according to Fischer's LSD at 5% significance level.

4.3.2.4 Root weight

There was a significant (P<0.05) difference in root weight of maize plants treated with the different endophytes and their mixtures (Table 4.7). Of all four bacterial mixtures, the *M. odoratus* 6G NYL18 + *A. faecalis* NYS7 + *Ralstonia* spp. NYR8 mixture had the highest root weight in both experiments (Table 4.7). Maize plants treated with all three endophyte mixtures had a mean root weight higher than the uninoculated control. In both experiments, there were no significant differences in the mean root weight of maize plants treated with *Ralstonia* spp. NYR8 and the mixture of *M. odoratus* 6G NYL18 + *A. faecalis* NYS7 + *Ralstonia* spp. NYR8

Table 4.7 Mean root weight (g) of maize plants after a growing period of six weeks under greenhouse conditions.

Treatment	Exp. 1	Exp. 2
CONTROL (Uninoculated control)	9.33b	10.67ab
A. faecalis NYS7	8.33bc	8.67bc
Ralstonia spp. NYR8	11.33a	10.33ab
M. odoratus 6G NYL18	9.67b	11.33a
M. odoratus 6G NYL18 + *Ralstonia* spp. NYR8	9.67b	8.67bc
Ralstonia spp. NYR8 + *A. faecalis* NYS7	7.33cd	7.33c
M. odoratus 6G NYL18 + *A. faecalis* NYS7	8.33bc	8.00c
M. odoratus 6G NYL18 + *A. faecalis* NYS7 + *Ralstonia* spp. NYR8	12.00a	12.00a
C + P (Pathogen inoculated control)	6.67d	7.00c
P value	<.001	<.001
L.S.D	1.36	1.98
S.E.D	0.64	0.94
CV%	8.6	12.3

*Mean root weight (g) of three replicates per treatment. Values with the same letters have no significant difference in the same column according to Fischer's LSD at 5% significance level.

4.3.2.5 Shoot weight

The mean shoot weight of maize plants treated with different bacterial endophytes against root rot caused by *Pythium* spp. is presented in Table 4.8. There were significant ($P<0.05$) differences between the treatments. The combination of all three bacterial endophytes increased the shoot weight compared to other bacterial mixtures. Even though the mixture of all three bacterial endophytes increased shoot weight, there were no significant differences between *Ralstonia* spp. NYR8 and *M. odoratus* 6G NYL18 + *A. faecalis* NYS7 + *Ralstonia* spp. NYR8. In relation to the negative control (C + P), seed treatment of maize plants with the bacterial isolates increased shoot weight.

Table 4.8: Shoot weight (g) of maize plants treated with different bacterial endophytes against root rot caused by *Pythium spp*.

Treatment	**Exp. 1**	**Exp. 2**
CONTROL (Uninoculated control)	71.67a	74.33a
A. faecalis NYS7	62.33b	63.33bc
Ralstonia spp. NYR8	64.67b	65.67b
M. odoratus 6G NYL18	62.33b	59.33c
M. odoratus 6G NYL18 + *Ralstonia* spp. NYR8	47.67c	45.67d
Ralstonia spp. NYR8 + *A. faecalis* NYS7	47.00c	49.67d
M. odoratus 6G NYL18 + *A. faecalis* NYS7	48.00c	49.00d
M. odoratus 6G NYL18 + *A. faecalis* NYS7 + *Ralstonia* spp. NYR8	62.33b	58.00c
C + P (Pathogen inoculated control)	30.33d	30.33e
P value	<.001	<.001
L.S.D	5.49	5.08
S.E.D	2.59	2.39
CV%	5.8	5.3

*Mean shoot weight (g) of three replicates per treatment. Values with the same letters have no significant difference in the same column according to Fischer's LSD at 5% significance level.

4.3.3 Potential mode of action

Bacterial endophytes used in this study did not produce chitinase (Table 4.9). Of the six bacterial endophytes, *B. cereus* NYR11, *P. mirabilis* NYR9 and *M. morganii* DG56-16 NYS3 against *R. solani* and *M. odoratus* 6G NYL18, *A. faecalis* NYS7 and *Ralstonia* spp. against *Pythium* spp., only *Ralstonia* spp. NYR8 did not produce cellulase and siderophores. Two bacterial endophytes (*P. mirabilis* NYR9 and *M. odoratus* 6G NYL18) did not produce protease, while all others did.

Table 4.9 Production of enzymes and siderophores by bacterial endophytes used in the greenhouse experiments.

Endophyte	Protease	Chitin	Cellulase	Siderophores
M. morganii DG56-16 NYS3	+ (6cm)	-	+ (4cm)	+ (2cm)
Alcaligenes faecalis NYS7	+ (7cm)	-	+ (4cm)	+ (4cm)
Ralstonia spp. NYR8	+ (2cm)	-	- (0cm)	- (0cm)
P. mirabilis NYR9	- (0cm)	-	+ (3cm)	+ (2cm)
Bacillus cereus NYR11	+ (6cm)	-	+ (4cm)	+ (3cm)
M. odoratus 6G NYL18	- (0cm)	-	+ (4cm)	+ (5cm)

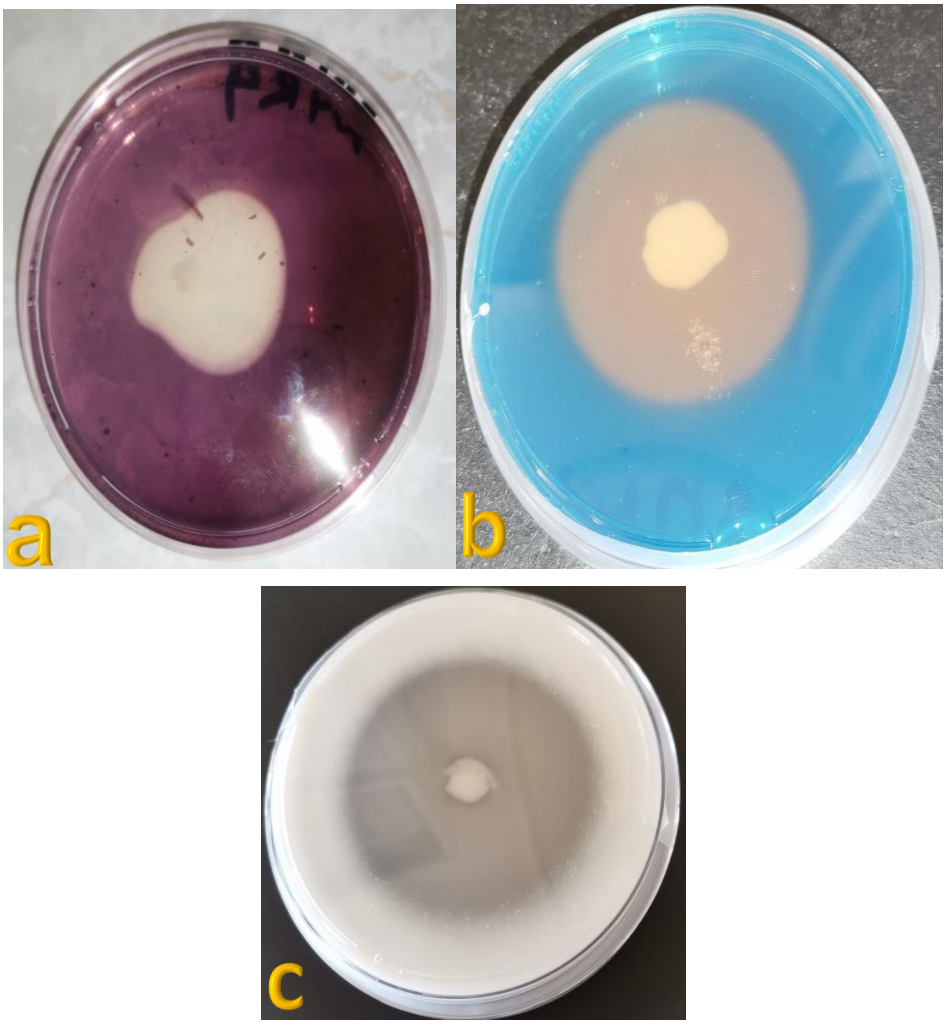


Figure 4.3: Results of the endophytes from the screening of potential modes of action. (a) Cellulase activity of bacterial endophyte *P. mirabilis* NYR9. (b), siderophores production of bacterial isolate *M. odoratus* NYL18 and (c) protease activity of bacterial endophyte *B. cereus* NYR11.

4.4 Discussion

Combining all three bacterial isolates *M. odoratus* 6G NYL18 + *A. faecalis* NYS7 + *Ralstonia* spp. NYR8 significantly increased the root weight and reduced the number of root lesions compared to other bacterial combinations. Out of all endophyte mixtures, *M. odoratus* 6G NYL18 + *A. faecalis* NYS7 + *Ralstonia* spp. NYR8 was the better combination even though single application of *M. odoratus* 6G NYL18 reduced root rot symptoms slightly better than this mixture. Bacteria have been found to produce secondary metabolites which induce antagonistic activities in their host plant and promote plant growth (Sulley *et al*., 2021).

It has been predicted that combining microbial agents as a treatment to plant diseases may lead to increased resistance levels against plant pathogens due to the combined antagonistic effect from the microorganisms (Xu *et al*., 2011). From this experiment, seed treatment with bacterial combinations of *P. mirabilis* NYR9 with *M. morganii* DG56-16 NYS3 and *B. cereus* NYR11 with *M. morganii* DG56-16 NYS3 against *R. solani* increased root length and weight in comparison to other bacterial combinations. Treatment with these two bacterial combinations also reduced the mean number of root lesions. *M. morganii* DG-16 NYS3 reduced root rot symptoms compared to other single applications of *P. mirabilis* NYR9 and *B. cereus* NYR11. Karthika *et al*., (2020) reported that *B. cereus* KTMA4 inhibits *F. oxysporum* and *Alternaria solani* in tomato plants by producing cellulase, protease, amylase, lipase, xylanase and siderophores. This coincides with the results from this study, whereby, *B. cereus* NYR11 produced cellulase, protease, and siderophores as potential modes of action. In this study, *M. morganii* NYS3 was able to degrade protein, cellulose and produce siderophores. *P. mirabilis* NYR9 produced cellulase and siderophores. Therefore, the antifungal activity of *M. morganii* DG56-16 NYS3 + *B. cereus* NYR11 and *M. morganii* DG56-16 NYS3 may be due to the additive or synergistic effects of the enzymes and siderophores.

Compared to all other treatments, the single application of *M. odoratus* 6G NYL18 reduced symptoms of root rot caused by *Pythium* spp. This endophyte reduced the mean number of root lesions, increased root and shoot weight and promoted root length. In general, the ability of *Myroides* spp. to colonise their hosts and form biofilms as reported by Jacobs and Chenia, (2009) may have had a positive effect. The results also showed that *M. odoratus* 6G NYL18 has the ability to produce cellulase and siderophores. These enzymes have been reported to inhibit the growth of many fungal

pathogens including *Pythium* spp. which were in question in this experiment (Djuidje *et al*., 2022). This therefore indicates that this isolate has beneficial effect on the growth and development of crops through pathogen inhibition.

In a study conducted by Prabhukarthikeyan *et al*., (2014), combining *Beauveria bassiana* (B2) and *Bacillus subtilis* (EPC8) significantly reduced *Fusarium* wilt incidence in tomato (*Solanum lycopersicum* Mill). Disease incidence in tomato plants treated with the combined microorganisms was 6.92% while the individual application of *B. subtilis* EPC8 was recorded at 9.51%. The general assumption is that microbial consortia will adapt to different soil and environmental conditions since these conditions imitate the microbial communities (Abdeljalil *et al*., 2021). Literature suggests that a combination of BCAs may be more effective than individual application (Abdeljalil *et al*., 2021). However, in this study, the individual application of *M. odoratus* 6G NYL18 against *Pythium* spp. outperformed some bacterial mixtures in root length, root weight, shoot weight, and number of root lesions.

Bacterial endophytes use different mechanisms to control plant pathogens, they can directly kill the pathogen or enhance the resistance of the host plant through induced systemic resistance. Induced systemic resistance involves the production of metabolites with antagonistic characteristics, e.g. siderophores, hydrolytic enzymes, and hydrogen cyanide (Riaz, *et al*., 2021). Siderophores improve the efficacy of BCAs by removing iron from the pathogen, thereby preventing its' development and metabolic activity (Francesco and Baraldi, 2021). In this study, five of the six bacterial endophytes used were able to produce siderophores. Therefore, it is possible that these endophytes use siderophores as one of their modes of action. It has been reported that different hydrolytic enzymes work together in preventing plant disease development. Because chitin, protein and cellulose make up the cell wall of fungal pathogens, the production of enzymes that degrade these compounds contributes to the efficacy of the BCAs (Oztekin and Karbancioglu-Guler, 2021). Therefore, it is likely that these enzymes had a combined effect against the pathogens in this study, e.g. in *M. odoratus* 6G NYL18 + *A. faecalis* NYS7 + *Ralstonia* spp. NYR8 and *M. morganii* DG56-16 NYS3 + *B. cereus* NYR11 combinations.

All the bacterial endophytes evaluated in this study did not produce chitinase. This means chitinase production is not a possible mode of action used by these

endophytes. The endophytes possibly slowed down the growth of the pathogens by siderophore production, cellulose and protein degradation except *Ralstonia* spp. NYR8 which only produced protease as presented in Table 4.9.

The efficacy of biocontrol agents is influenced by the rhizosphere conditions (Guetsky *et al*., 2002). Therefore, it is likely that when environmental conditions favour the functioning of one mechanism, another might be counteracted (Guetsky *et al*., 2002). Hence in this study chitinases were not produced and other isolates inhibited the pathogen one experiment and in the other its efficacy was reduced or increased. This suggests that even though mixing *M. morganii* DG56-16 NYS3 with either *B. cereus* NYR11 or *P. mirabilis* NYR9 reduced *R. solani* root rot, when all three organisms were mixed together, mechanisms from some of the organisms were suppressed.

When *M. morganii* DG56-16 NYS3 is mixed with either *B. cereus* NYR11 or *P. mirabilis* NYR9, there seems to be synergism between the microorganisms. *Ralstonia* spp. NYR8 only produced protease as a mode of action while *M. odoratus* 6G NYL18 produced cellulase and siderophores. *A. faecalis* was able to degrade cellulose and protein, and produce siderophores, hence it had a low number of root lesions compared to other treatments except *M. odoratus* 6G NYL18 + *A. faecalis* NYS7 + *Ralstonia* spp. NYR8 in both experiments. The *M. odoratus* 6G NYL18 + *A. faecalis* NYS7 + *Ralstonia* spp. NYR8 mixture showed to have additive effects against *Pythium* spp. However, individual applications of *Ralstonia* spp. NYR8 and *M. odoratus* 6G against *Pythium* spp. were the best treatments.

A consortium of microorganisms does not necessarily increase plant resistance to diseases as seen on the evaluation of bacterial isolates against *Pythium* spp. Some of the bacterial combinations against *R. solani* reduced root rot symptoms and have potential to be used as BCAs. Other modes of action utilized by endophytes such as phosphorous solubilisation, production of antibiotics, nitrogen fixing abilities and biosurfactant activity were not evaluated in this study. Therefore, these bacterial endophytes might have other possible modes of action which were not evaluated in this study.

4.5 References

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Dissertation overview of the research findings and their implications and future research

Introduction

Maize (*Zea mays* L.) is an economically important crop worldwide. However, maize is susceptible to root rot pathogens such as *Pythium arrhenomanes* (Reyes-Tena *et al*., 2018) and *Rhizoctonia solani* (Boine *et al*., 2014). Root rot pathogens can cause yield losses of up to 100% in severe epidemics (Suleiman and Emua., 2009; Hooda *et al*., 2015). Plant diseases reduce yield quality and quantity (Mukanga *et al*., 2011); hence mitigation strategies are implemented. Common root rot management strategies include crop rotation, using resistant varieties and fungicides. Biological control is an alternative control strategy to prevent fungicide resistance and the negative effects of the fungicides on humans and the environment.

Endophytes are microorganisms that live asymptomatically within plant tissues. Endophytes are used as biocontrol agents (BCAs) since they produce antimicrobial compounds against pathogens. These can be siderophores or lytic enzymes e.g. cellulase, chitinase and protease. Plant pathogens and endophytes compete for nutrients and space within the host plant, reducing colonization by pathogens. Endophytes can induce systemic acquired resistance (SAR) in their host plants (Bahmani *et al*., 2021). Medicinal plants have been used against human pathogens, as they produce natural bioactive products. It has been suggested that endophytes isolated from medicinal plants may produce secondary metabolites with inhibitory effects against plant pathogens (Abdulazeez *et al*., 2020).

The aim of this study was to isolate endophytes from the medicinal plant *Arctotis arctotoides* (L.f) O. Hoffm and evaluate their antifungal activity against *R. solani* and *Pythium* spp. root rot pathogens of maize. The specific objectives were as follows: 1) To isolate bacterial endophytes from *A. arctotoides* and screen *in vitro* their potential to inhibit mycelial growth of *R. solani* and *Pythium* spp. 2) Evaluate selected bacterial endophytes as seed treatment on maize plants against *R. solani* and *Pythium* spp. in the greenhouse. 3) Evaluate mixtures of the best three endophytes against *R. solani* and *Pythium* spp. in the greenhouse and elucidate their potential modes of action.

Chapter 2: Isolation, -and *in vitro* screening of bacterial endophytes from *Arctotis arctotoides* (L.f.) O. Hoffm against two root rot pathogens, *Pythium* spp. and *Rhizoctonia solani*.

Major findings:

- A total of 26 bacterial endophytes were isolated from the leaves, stem, and roots of *A. arctotoides*.
- Ten (10) isolates were antagonistic to *R. solani*. Using the Internal Transcribed Spacers (ITS) sequencing, isolates were identified as *Bacillus* spp., *Morganella morganii*,*Proteus* spp., *Pseudomonas putida*, *Stenotrophomonas maltophilia*, and *Lysinibacillus pakistanensis*. The percentage inhibition of these bacterial strains against *R. solani* ranged from 17 - 50%.
- Eleven (11) isolates inhibited the growth of *Pythium* spp., and thes isolates were identified as *Morganella morganii*, *Ralstonia* spp., *Bacillus* spp., *Alcaligenes faecalis*, *Serratia marcescens*, *Pseudomonas putida*, and *Myroides odoratus*. The percentage inhibition against *Pythium* spp. ranged from 8 - 64%.
- *L. pakistanensis* NYL21, *P. mirabilis* NYR9, and *M. morganii* DG56-16 NYS3 inhibited mycelial growth of *R. solani* better than other isolates.
- *S. marcescens* NYS8, *M. morganii* AR_0133 NYL12, and *A. faecalis* NYS7 had higher percentage inhibition against *Pythium* spp.

Implications and future research

The antifungal activity of microorganisms *in vitro* may be different in greenhouse conditions as a result of the host, pathogen, endophyte and environmental conditions. Therefore, the potential of bacterial endophytes with good inhibition against *R. solani* and *Pythium* spp. *in vitro* can be evaluated under greenhouse conditions. Out of the 26 bacterial endophytes isolated in this study, seven were identified as strains of *M. morganii*. In this study, only a total of 26 bacterial endophytes were evaluated, in future studies, more bacterial endophytes can be included as this will also increase chances of isolating antagonistic endophytes. The scope for isolation can also be widen in terms of host plant varieties used, regions in which the plants are collected and also other medicinal plants may be considered.

Chapter 3: Evaluation of selected bacterial endophytes against maize root rot pathogens, *R. solani* and *Pythium* spp. under greenhouse conditions.

Major findings:

- The reduction of root rot symptoms by endophytes was not consistent in all experiments.
- Experiment 1: In maize plants inoculated with *R. solani*, *B. cereus* NYR11, *Bacillus* spp. NYR2, and *Proteus mirabilis* NYR9 were the best three endophytes with a mean plant height of 58.67cm, 55.00cm, and 52.67cm, respectively. However, in Experiment 2, *S. maltophilia* NYL15, *P. putida* NYR14, and *Morganella morganii* KC-Tt-01 NYL20 were the best three treatments at 108.67cm, 99.67cm and 99.67cm respectively in Week 6.
- The mean root length of maize plants treated with *S. maltophilia* NYL15 in the presence of *R. solani* was higher than the mean root length of other treatments except for the uninoculated control in Experiment 1. In Experiment 2, treatment with *M. morganii* DG56-16 NYS3 resulted in a higher mean root length than all other treatments including the uninoculated control.
- In both experiments, treatment of maize plants with *P. mirabilis* NYR9 significantly ($P<0.05$) reduced the mean number of root lesions by 56.28% and 46.21%, respectively, compared to the pathogen inoculated control.
- The mean root weight of maize plants treated with *M. morganii* DG56-16 NYS3 was higher than all other treatments in Experiment 1. *M. morganii* L143 NYR3 increased the mean root weight of maize plants in Experiment 2 compared to all other treatments.
- The shoot weight of *B. cereus* NYR11 and *S. maltophilia* NYL15 treated maize plants in Experiments 1 and 2, respectively, was the highest shoot weight amongst the different treatments.

- For *Pythium* spp. inoculated maize plants, treatment with *M. odoratus* 6G NYL18 had the best plant height (108.67cm and 106.00cm) in Week 6, compared to all other treatments in Experiment 1 and 2 respectively. Seed

treatment with the bacterial endophytes significantly ($P<0.05$) increased plant height in both experiments.

- In both experiments, *M. morganii* AR_0133 NYL12 had the highest root length with a mean of 49.00cm and 42.67cm. Root length of maize plants treated with this endophyte was also higher than the uninoculated control.
- Compared with the pathogen inoculated control, *A. faecalis* NYS7 decreased the number of root lesions by 26.70% and 82.36% in Experiments 1 and 2, respectively. *Ralstonia* spp. NYR8 reduced the number of root lesions by 33.51% and 52.91% in Experiments 1 and 2, respectively.
- The average root weight of maize plants treated with *M. morganii* AR_0133 NYL12 was 13.0g and 12.0g in Experiments 1 and 2, respectively.
- Compared with the pathogen inoculated control, treatment with *M. odoratus* 6G NYL18 resulted in a higher shoot weight than other treatments in both experiments.

Implications and future research

Some of the bacterial endophytes in this study reduced root rot symptoms in *R. solani* and *Pythium* spp. inoculated plants. In this study, bacterial endophytes such as *M. morganii* DG56-16 NYS3, *B. cereus* NYR11, *M. morganii* L143 NYR12 and *M. odoratus* 6G NYL18, increased plant height of maize plants compared to the pathogen inoculated control in the presence of *R. solani* and *Pythium* spp. Bacterial endophytes such as *P. mirabilis* NYR9, *A. faecalis* NYS7, and *Ralstonia* spp. NYR8, reduced the mean number of root lesions compared to the pathogen inoculated control. In future studies, efficacy of the different endophytes using various quantities and endophyte concentrations can be evaluated. The efficacy of the bacterial endophytes might vary at different concentrations.

Chapter 4: Mixtures of bacterial endophytes as seed treatment against *Pythium* spp. and *R. solani* in maize under greenhouse conditions.

Major findings:

- In Experiment 1 (*R. solani*), *P. mirabilis* NYR9 + *M. morganii* DG56-16 NYS3 had the highest height (105cm) compared to all other treatments, whereas *B. cereus* NYR11 + *M. morganii* DG56-16 NYS3 was the best mixture (100cm) in Experiment 2.
- In *R. solani* inoculated plants, mixtures of *P. mirabilis* NYR9 with *M. morganii* DG56-16 NYS3 and *B. cereus* NYR11 with *M. morganii* DG56-16 NYS3 reduced the mean number of root lesions, increased root length, root and shoot weight compared to the pathogen inoculated control and other mixtures.

- In Experiment 1 and 2, *M. odoratus* 6G NYL18 + *Ralstonia* spp. NYR8 had the highest height (103cm) and (105cm), respectively, compared to other mixtures in *Pythium* spp. inoculated plants.
- Compared to all other bacterial mixture applications, *M. odoratus* 6G NYL18 + *A. faecalis* NYS7 + *Ralstonia* spp. NYR8 is the only mixture that reduced root rot symptoms in the presence of *Pythium* spp.
- In terms of root length, single application of *M.odoratus* 6G NYL18 had the highest root length followed by *M. odoratus* 6G NYL18 + *A. faecalis* NYS7 + *Ralstonia* spp. NYR8.
- *M. odoratus* 6G NYL18 + *A. faecalis* NYS7 + *Ralstonia* spp. NYR8 reduced the mean number of root lesions compared to other mixtures.
- In Experiment 1, *M. odoratus* 6G NYL18 + *A. faecalis* NYS7 + *Ralstonia* spp. NYR8 increased root weight, but there were no significant ($P>0.05$) differences between this mixture and the single application of *M. odoratus* 6G NYL18 in Experiment 2, as both treatments increased root weight more than the other treatments.
- There were no significant ($P>0.05$) differences in shoot weight of *M. odoratus* 6G NYL18 + *A. faecalis* NYS7 + *Ralstonia* spp. NYR8 and *M. odoratus* 6G NYL18. However, *Ralstonia* spp. NYR8 had a higher shoot weight than *M.*

odoratus 6G NYL18 + *A. faecalis* NYS7 + *Ralstonia* spp. NYR8 and *M. odoratus* 6G NYL18.

- Of all the bacterial endophytes used in this study, only *Ralstonia* spp. NYR8 did not produce cellulase and siderophores. *P. mirabilis* NYR9 and *M. odoratus* 6G NYL18 did not produce protease.

Implications and future research

Even though using microorganism consortiums is considered superior to single applications as different modes of action will be integrated (Abdeljalil *et al*., 2021), this was not the case for some microorganisms used in this study. Considering the results obtained from this study, in some of the parameters, *M. odoratus* 6G NYL18 or *Ralstonia* spp. NYR8 were better as single treatments than in mixtures. This may be attributed to the fact that when microorganisms are used in mixtures, the one organism might suppress certain traits of the other (Guetsky *et al*., 2002). Bacterial mixtures that reduced root rot symptoms might have done so because of the additive or synergistic effects when mixed together. *B. cereus* NYR11 + *M. morganii* D56-16 NYS3 or *P. mirabilis* NYR9 + *M. morganii* DG56-16 NYS3 may be used as BCAs against *R. solani* root rot of maize. *M. odoratus* 6G NYL18 + *A. faecalis* NYS7 + *Ralstonia* spp. NYR8 may be used against *Pythium* spp. root rot. However, single application of *M. odoratus* 6G NYL18 is preferred. The potential modes of action of the isolates used in this study include siderophore production, protein, and cellulose degradation. Other modes of action, such as nutrient competition could be evaluated to better understand how these endophytes inhibit pathogen growth.

Way forward

In this study, some positive results were obtained by the use of the endophytes isolated from *A*. *arctotoides* in the management of *R. solani* and *Pythium* spp. root rot in maize. Further research to increase the extent of sources of bacterial endophytes from closely related plants will enhance the variety of isolates and possibly more active and efficient bacterial endophytes against the root rot pathogens. Moreover, the

search can also include fungal endophytes. It is also important that the best isolates in future work should be studied in terms of endophytic colonisation of maize plants using molecular methods. Where, and how far into the maize plant the bacterial endophyte(s) can move from the point of application will be essential in mechanisms such as signalling of induced systemic resistance (ISR) elicited in plants and thus priming resistance in maize plants. It is also important that the study is extended to include field studies to determine the efficacy of the best isolates under field conditions that mimic large scale, commercial maize cultivation.

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